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A nominee must be a resident of the United States or Canada. During the period covered by the nomination, he shall have been actively engaged in, have completed or have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, he shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award, consisting of $1,000 and a diploma setting forth the reasons for selection of the recipient, will be presented at the annual meeting of the ASSOCIATION. At this meeting, the recipient will deliver a paper or lecture upon the subject of his scientific work which will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is made is provided for up to $250.

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The award is now in its eighth year. The recipients of the award in the order named are: Dr. William T. Salter, Yale University pharmacologist; Dr. George M. Curtis, Department of Surgical Research, Ohio State University; Dr. C. P. Leblond, McGill University, Montreal, Canada; Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Veterans Hospital, Houston, Tex., jointly; Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri; Dr. Jack Gross, State University of New York, College of Medicine in Brooklyn; and Dr. Domenick Papa, Schering Corporation, Bloomfield, N. J.

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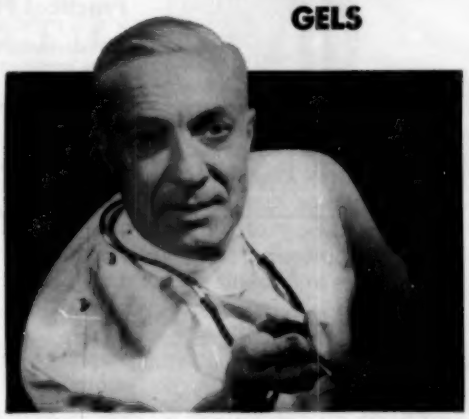

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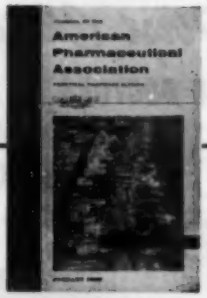

A Preview of Advertising

In the September 1955 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ **Abbott Laboratories** has three insertions: one announces **Placidyl,** *an important new non-barbiturate hypnotic;* another emphasizes the efficacy of **Sur-Bex with C** as a dietary supplement; and still another is given over to Abbott's potent antibiotic against coccic infections, **Erythrocin.**

▸ Pointed up by the **Ames Company** are **Aminet,** *for rapid relief in asthma,* and **Diatussin,** *drop dosage cough control,* products of the advertiser's **Bischoff Division.**

▸ **Span, Tween, Arlacel,** and **Sorbo** are some of the adjuncts offered by **Atlas Powder Company** for use in pharmaceutical preparations.

▸ In its familiar position on the third cover, **Hoffmann-La Roche Inc.** repeats two special offers concerning its antacid, **Syntrogel,** and **Supradin,** its therapeutic multivitamin formula.

▸ Useful in the treatment of dysmenorrhea are **Lutrexin Tablets,** product of **Hynson, Westcott & Dunning.**

▸ **Lederle Laboratories'** graphic presentation stresses the gains to be realized from effective display and merchandising of its products.

▸ Opposite the Table of Contents appears "a distinguished member of the **Lilly** family of vitamins," **Vi-Mix Drops.**

▸ Described as being "virtually without side effects," is a new antiarthritic, **Salcort,** product of **The S. E. Massengill Company.**

▸ The *new* **Merck Inverted Rx Display Set** offered pharmacists is designed to enhance the professional appearance of the pharmacy.

▸ Attention is directed by the **Owens-Illinois** announcement to the *visibility* of its **Duraglas** *Clear Dry Rx Squares.*

▸ Of **Siblin Tablets,** for uncomplicated constipation, and useful in curbing appetite, **Parke, Davis** says its "multiple uses multiply sales."

▸ *Be sales fortified,* suggests **Pfizer,** with **Tetracyn SF,** providing simultaneous antiinfective and nutritional therapy in a single prescription.

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▸ A *new* display carton for its **Benzedrex Inhaler** headlines the current **Smith, Kline & French** announcement.

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▸ Virtually synonymous with the condition it treats is **Upjohn's Kaopectate** for diarrhea.

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▸ Sounding a note of advice, **Winthrop-Stearns** suggests ordering full stocks *now* on **Creamalin** and **Tricreamalate,** its antacids.

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out September 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *SEPTEMBER 1955* — NUMBER 9

CONSECUTIVE NO. 18

Inhibition of Hydrolysis of Esters in Solution by Formation of Complexes I*

Stabilization of Benzocaine with Caffeine

By TAKERU HIGUCHI and LEON LACHMAN

A new approach to possible preservation of drugs undergoing hydrolytic degradation is presented. It is shown that the rate of hydrolysis of benzocaine in aqueous solution can be substantially inhibited by addition of caffeine which has previously been shown to complex with this drug. Presence of two and one-half per cent of caffeine in the system resulted in reduction of the hydrolytic rate to less than one-fifth of that in absence of the xanthine. Mathematical analysis of the results obtained indicates that the complexed form of the drug does not undergo, surprisingly, any perceptible cleavage at the ester linkage.

ALTHOUGH DETERIORATION of most pharmaceutical preparations possibly occurs through *hydrolytic* mechanisms, no suitable method of suppressing these reactions by use of chemical additives has been proposed. The principal avenues of attack open in the past to pharmaceutical formulators in increasing the stability of drugs susceptible to hydrolytic breakdown have been to utilize the drug in a form of insoluble suspension, and to minimize the concentration of the catalytic species, usually hydrogen or hydroxyl ions. It is the purpose of this paper to present our recent experimental findings that a significant degree of stabilization may also be produced under certain instances by addition of a complexing agent to pharmaceuticals undergoing hydrolytic cleavage. Specifically, it is shown that benzocaine in a benzocaine-caffeine complex, a water-soluble molecular compound which forms quite readily in solutions, cleaves at an insignificant rate as compared to benzocaine itself.

PAST WORK

The concept of using chemical stabilizers as aids in preserving pharmaceuticals is by no means novel. Terms such as stabilizers, preservatives, inhibitors, retarders, antioxidants, etc., which enjoy wide pharmaceutical usage are in themselves evidence of this approach to pharmaceutical stabilizations. Yet, aside from substances which operate through *p*H changes, no earlier study seems to have been made on possible utilization of chemical additives in inhibiting the rate of hydrolytic breakdown in contrast to oxidation-induced degradation of pharmaceuticals in solution. The stabilizers of the past have been largely restricted in their usage to deterioration reactions caused by free radical mediated chain reactions or through biological processes. Small traces of antioxidants are thus often used to preserve a system against polymerization or oxidative breakdown. A low concentration of mercurial can prevent deterioration through microbiological action. On the other hand, the field of

* Received April 4, 1955, from the School of Pharmacy, University of Wisconsin, Madison. Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.

Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

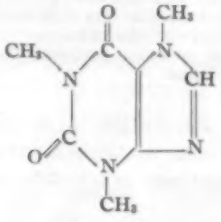
chemical preservation against hydrolytic (or any other molecular) reactions is comparatively unexplored.

The notion of drug stabilization through complex formation is probably new. As mentioned in the introduction, although one of the most successful methods of reducing the rate of deterioration of drugs sensitive to hydrolytic loss is based on conversion to a slightly soluble dispersed form, this does not in the true sense involve complex formation. The possibility of utilizing complex formation to inhibit hydrolytic rates of materials *in solution*, furthermore, has not been considered previously by anyone.

POSSIBLE MECHANISM BY WHICH COMPLEX FORMATION MAY AFFECT HYDROLYTIC RATE

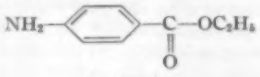
It is extremely difficult on the basis of present-day knowledge of chemical mechanisms and molecular interaction to postulate *a priori* the possible effect of complex formation on reaction rates. Although the catalytic action of hydrogen ion on ester hydrolysis is explicable on the basis of formation of an intermediate activated complex, the mechanism of action, for example, of hydrolytic enzyme on esters is far from being elucidated. It is reasonable, nevertheless, to expect some modification of the hydrolysis rates of carboxylic esters if they were to complex with a highly polarized compound, especially if the ester functions were involved in the complex formations.

The hydrolytic rates may be influenced in two ways by such complex formation. The effect may be of steric nature. Obviously the attachment of a large caffeine molecule,



Caffeine

for example, on a benzocaine molecule,



Benzocaine

can greatly modify the frequency and ease of encounter of the ester with various catalytic species. The reaction rate may also be affected by electronic influence of the complexing agent. Thus, for example, the affinity of the ester carbonyl for hydroxyl ion may be considerably altered by the near presence of the xanthine. In general, the steric effect would be expected to decrease the hydrolytic rate, whereas, the electronic effect may increase or decrease the reaction velocity.

COMPLEXING BEHAVIOR OF BENZOCAINE

The possible influence of complex formation on rates of hydrolytic breakdown was tested in this investigation on benzocaine since the drug has a relatively simple chemical structure and its degradation products are known. Moreover, since it forms a water-soluble complex with caffeine, it provided a suitable system for the study of the influence of complex formation on the kinetics of ester hydrolysis.

Higuchi and Lach (1) have already shown that benzocaine complexed strongly with caffeine giving a water-soluble complex composed apparently of one molecule each of benzocaine and caffeine. The stoichiometry of the complexing reaction was computed from the increases in the solubilities of both benzocaine and caffeine resulting from complex formation. It was shown that the equilibrium constant, A

$$A = \frac{[\text{Benzocaine-Caffeine}]}{[\text{Benzocaine}]\,[\text{Caffeine}]} \qquad (1)$$

for the apparent reaction

$$\text{Benzocaine} + \text{Caffeine} = \text{Benzocaine-Caffeine} \qquad (2)$$

over a range of concentrations of both caffeine and benzocaine remained essentially constant with an average value of 60 L./moles at 30°.

More recent investigation has given evidence indicating that caffeine in aqueous solutions even in these dilutions may dimerize significantly. Nevertheless, the extent of benzocaine binding by caffeine appears to be still effectively expressed by relationships (1) and (2).

RESULTS AND DISCUSSION

In order to determine the effect of the complexing action of caffeine on the rate of hydrolytic breakdown of benzocaine, the chemical kinetics of the hydrolytic reaction was studied experimentally in absence and presence of the complexing agent.

The results of a rate study on the hydrolysis of benzocaine in water free from caffeine are shown in Figs. 1 and 2 and in Table I. These hydrolytic reactions were carried out at 30° in water and were catalyzed with OH^- in form of barium hydroxide. Barium hydroxide solution was used in order to minimize interference from carbonate formation.

TABLE I.—EFFECT OF HYDROXYL ION CONCENTRATION ON THE HALF-LIFE OF BENZOCAINE IN AQUEOUS $Ba(OH)_2$ SOLUTIONS AT 30° C.

Hydroxyl Ion Concentration	Half-Life (From Fig. 1)
0.02 *N*	139.0 min.
0.04 *N*	69.0 min.
0.06 *N*	44.5 min.

The residual concentration of benzocaine in barium hydroxide solution at different time intervals is shown in Fig. 1. It is evident from the figure that the rate of hydrolysis of benzocaine at 30° with varying amounts of constant hydroxyl ion concentration is first order with respect to the ester concentration. Figure 2 shows that the reaction rate of the hydrolysis is in direct proportion to the hydroxyl ion concentration. Thus, the overall reaction between benzocaine and hydroxyl ion was found to be second order according to the rate equation,

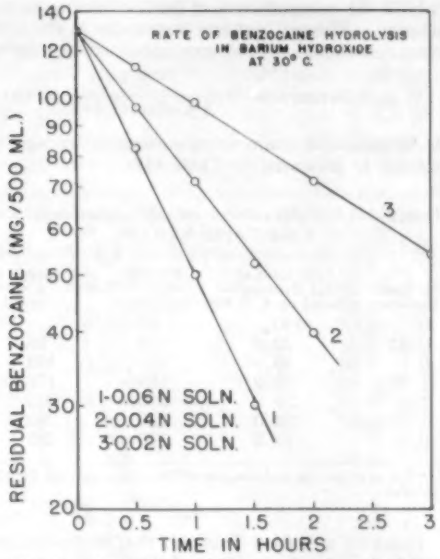

RATE OF BENZOCAINE HYDROLYSIS IN BARIUM HYDROXIDE AT 30° C.
RESIDUAL BENZOCAINE (MG./500 ML.)
TIME IN HOURS
1-0.06 N SOLN.
2-0.04 N SOLN.
3-0.02 N SOLN.

Fig. 1.—Hydrolysis of benzocaine in solution at varying hydroxyl ion concentrations [$Ba(OH)_2$] and at 30° C.

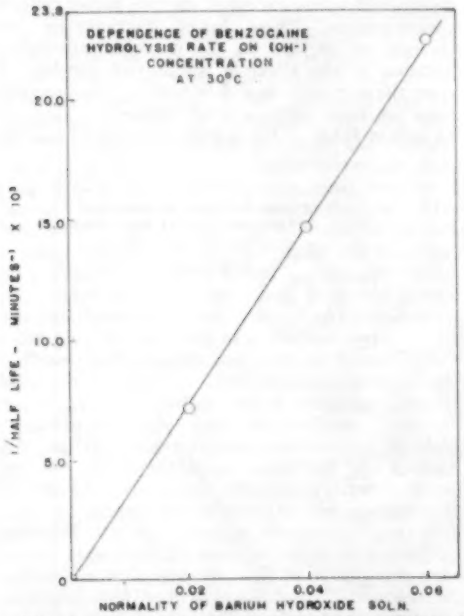

DEPENDENCE OF BENZOCAINE HYDROLYSIS RATE ON (OH⁻) CONCENTRATION AT 30°C
1/HALF LIFE - MINUTES⁻¹ X 10³
NORMALITY OF BARIUM HYDROXIDE SOLN.

Fig. 2.—A plot showing the catalytic activity of hydroxyl ions upon the rate of hydrolytic degradation of benzocaine at 30° C.

$$\frac{d[\text{Benzocaine}]}{dt} = -k[\text{Benzocaine}]\,[OH^-], \quad (3)$$

in agreement with the behavior of carboxylic esters in general.

No earlier kinetic study seems to have been made on the hydrolysis of benzocaine in water. Ingold and Nathan (2) studied the reaction, but employed hydroalcoholic systems to solubilize the drug. From their data it appears that the second order rate constant k in Eq. 3 is approximately equal to 2.1×10^{-3} L./Gm. mole/sec. at 30° and for a solvent system composed of 85% ethanol and 15% water. In contrast, the results of the present investigation indicate a value of 4.2×10^{-3} L./Gm. mole/sec. for runs made in water as the solvent, a difference not completely explicable on the basis of difference in water concentration and dielectric constant alone.

The influence of caffeine on the hydrolysis rate of benzocaine in water is shown in Fig. 3 and Table II.

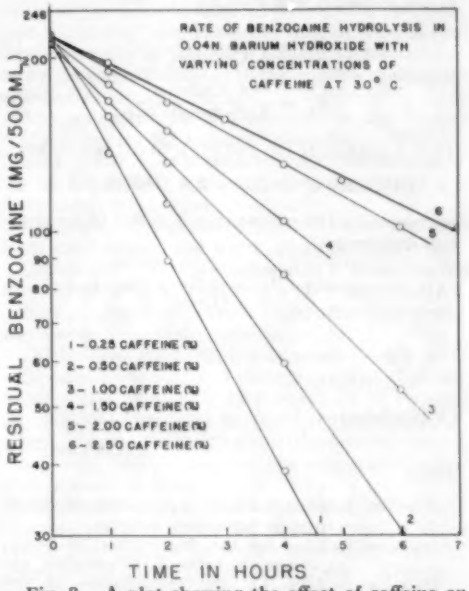

RATE OF BENZOCAINE HYDROLYSIS IN 0.04N. BARIUM HYDROXIDE WITH VARYING CONCENTRATIONS OF CAFFEINE AT 30° C.
RESIDUAL BENZOCAINE (MG./500ML.)
TIME IN HOURS
1 - 0.25 CAFFEINE (%)
2 - 0.50 CAFFEINE (%)
3 - 1.00 CAFFEINE (%)
4 - 1.50 CAFFEINE (%)
5 - 2.00 CAFFEINE (%)
6 - 2.50 CAFFEINE (%)

Fig. 3.—A plot showing the effect of caffeine on the rate of hydrolytic degradation of benzocaine at 30° C. and at 0.04 N hydroxyl ion concentration [$Ba(OH)_2$].

TABLE II. THE INFLUENCE OF CAFFEINE ON THE HALF-LIFE OF BENZOCAINE IN 0.04 N $Ba(OH)_2$ SOLUTIONS AT 30° C.

Per Cent Caffeine Used	Half-Life (From Fig. 3)
0.0	69 min.
0.25	95 min.
0.5	125 min.
1.0	180 min.
1.5	230 min.
2.0	320 min.
2.5	370 min.

The rates were determined in aqueous barium hydroxide solution at 30° containing a hydroxyl ion concentration of 0.04 N. The hydroxyl ion concentration of 0.04 N was chosen so that a run could readily be completed within a twelve-hour period.

It is apparent from Table II that the rate of ester breakdown is strongly repressed by the addition of caffeine. This can only be ascribed to the slower

rate of breakdown of the complexed ester as compared to the free benzocaine. Thus we can write

$$k = k_f F_f + k_c F_c \quad (4)$$

where

k = the apparent rate constant for the degradation of benzocaine in presence of caffeine,
k_f = the rate constant for the degradation of uncomplexed benzocaine,
k_c = the rate constant for the degradation of the complexed benzocaine,
F_f = the fraction of total benzocaine remaining free in solution and finally,
F_c = the fraction of total benzocaine in form of the complex in solution.

It can be readily seen from Eq. (1) that

$$F_f = \frac{1}{A[\text{Caffeine}] + 1} \quad (5)$$

$$F_c = \frac{A[\text{Caffeine}]}{A[\text{Caffeine}] + 1} \quad (6)$$

Then substituting (5) and (6) into (4), we obtain

$$(A[\text{Caffeine}] + 1)k = k_c A[\text{Caffeine}] + k_f \quad (7)$$

or expressing the relationship in terms of reciprocal half-lives we get

$$(A[\text{Caffeine}] + 1)\frac{1}{t^1/_2} = \frac{1}{(t^1/_2)_c} A[\text{Caffeine}] + \frac{1}{(t^1/_2)_f} \quad (8)$$

or

$$(A[\text{Caffeine}] + 1)\frac{1}{t^1/_2} - \frac{1}{(t^1/_2)_f} = \frac{1}{(t^1/_2)_c} A[\text{Caffeine}] \quad (9)$$

where

$t^1/_2$ = half-life for the degradation of benzocaine in presence of caffeine,
$(t^1/_2)_c$ = half-life for the degradation of benzocaine in the form of the complex, and
$(t^1/_2)_f$ = half-life for the degradation of free benzocaine.

Since the value of the effective equilibrium constant A is approximately known from the work of Higuchi and Lach (1), it was possible to make an estimate of the k_c or $1/(t^1/_2)_c$ from any rate value obtained for the degradation of benzocaine in solution containing caffeine. For the system containing 1% caffeine (Table III), which was 0.04 N with respect to OH^- concentration and at 30°, however, we found that k_c was approximately zero.

From this and other calculations it appeared that the reaction rate of the complexed ester was, at the most, extremely small as compared to that of the free form. Thus, for all practical purposes Eq. 9 reduces to

$$(A[\text{Caffeine}] + 1)\frac{1}{t^1/_2} = \frac{1}{(t^1/_2)_f} \quad (10)$$

This low reactivity of the complex is even more evident in Fig. 4, where the per cent free benzocaine, at the various concentrations of caffeine, have been plotted against the reciprocal half-life of the solutions. The straight line passing through the origin again shows that the reaction rate of the hydrolysis is directly proportional to and a function only of the concentration of free benzocaine in the solution. The per cent free beuzocaine in the solutions containing caffeine was calculated as follows:

$$\%\ \text{Free Benzocaine} = \frac{1}{A[\text{Caffeine}] + 1} \times 100$$

A correlation of results using various percentages of caffeine is presented in Table III.

TABLE III.—COMPARISON OF EXPERIMENTAL AND CALCULATED RESULTS

Per Cent Caffeine	Per Cent of Free Benzocaine (Based on A = 60)	Experimental $t^1/_2$ (min.)	Calculated on Basis (A = 60) $t^1/_2$ (min.)
0	100	69	...
0.25	72.3	95	96
0.5	56.0	125	123
1.0[a]	37.0	180	176
1.5	29.2	230	229
2.0	23.0	320	282
2.5	19.3	370	335

[a] See text for the calculation of the rate constant for the complex.

It can be seen from Table III that the calculated half-lives on the basis (1) that the complexed drug did not degrade and (2) that the stability constant of the complex was effectively equal to 60 were in reasonable agreement with the experimentally determined values except at the two higher caffeine concentrations. These latter discrepancies are thought to be due to the observed substantial variance of the effective equilibrium constant A from the average value of 60 used in these calculations at these caffeine concentrations. This can be seen in Table IV taken from the paper of Higuchi

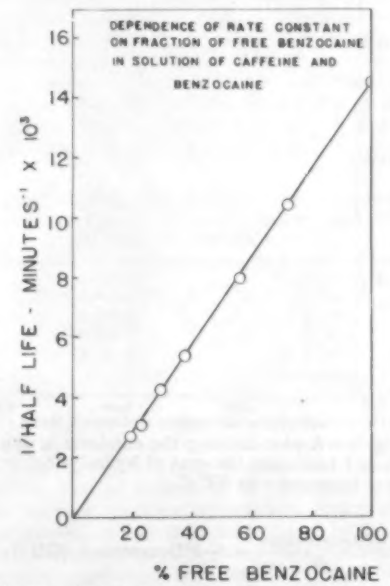


Fig. 4.—A plot showing the reaction rate of the hydrolysis to be directly proportional and a function only of the concentration of free benzocaine in solution.

and Lach (1). The longer observed half-life at higher caffeine concentrations, moreover, cannot be ascribed to the hydrolytic breakdown of the complexed drug since this would result in the opposite effect, i. e., shorter half-life.

TABLE IV.—EFFECTIVE STABILITY CONSTANT OF BENZOCAINE-CAFFEINE COMPLEX AT 30° IN WATER[a]

Caffeine Added to System Concn. × 10^2	Effective Equilibrium Constant
0.000	...
1.066	53.2
3.188	51.7
5.410	56.6
6.339	63.7
7.271	56.7
8.332	53.2
9.426	58.3
9.443	55.9
10.634	53.4
11.769	61.5
13.003	59.8
14.087	65.1
14.673	62.4
15.940	65.3
16.489	62.4
17.162	64.8
17.862	63.6

[a] From data of Higuchi and Lach (1).

Although the complex does not appear to undergo any detectable degradation, it does not mean, of course, that the complexed ester is completely immune toward the hydrolytic reaction, but rather that the experimental method employed was too insensitive to pick up this apparently very slow reaction in presence of the relatively fast cleavage reaction of the free ester. Nevertheless, the data suggest that the complexed ester must react at a rate at least as slow as 1/100 that of the free. This is based on the fact that the intercept on the left *y* axis in Fig. 4 is at least that much less than that on the right axis. This is still a rather interesting conclusion. It is somewhat surprising that a mild molecular reaction of this type should result in such a total loss of reactivity at a covalent bond.

These results suggest that complex formation can be an aid in the preservation of many aqueous pharmaceutical preparations which undergo deterioration through hydrolytic mechanisms. Thus at 2.5% caffeine concentration approximately $5^1/_2$-fold increase in the half-life of benzocaine was induced by the addition of the complexing agent. Moreover, the information obtained in this investigation indicated that if a stronger complexing agent of a similar type could be found, it might be possible to inhibit the hydrolysis reaction by complexing substantially all of the drug in solution. It is apparent, however, that there is still much to be learned concerning the effects of complex formation on the stability of pharmaceuticals. Many more compounds must be studied before any statement can be made as to the general effect of complex formation on the stability of pharmaceuticals.

EXPERIMENTAL

For the purpose of the present study, it was desirable to develop an analytical method which would permit differentiation of undegraded benzocaine from its degradation products and caffeine. The feasibility of the chromatographic technique for this purpose is evident from the chromatogram shown in Fig. 5. The partition systems employed in this investigation consisted of an aqueous internal phase held on silicic acid and a mobile phase of diisopropyl ether. The nonpolar solvent diisopropyl ether removed the undegraded benzocaine, whereas, the caffeine and degradation products remained on the silicic acid column in the internal water phase. Since the sample to be analyzed was in aqueous solution, it was mixed with dry silicic acid and added to the column in a form of a slurry dispersed in Skellysolve®-C. Some interaction appears to take place between the solution of caffeine and benzocaine in the aqueous internal phase. In the solution containing benzocaine and caffeine we find the benzocaine eluted from the column in fractions 4 and 5 instead of 3 and 4 as in the solution containing only benzocaine.

The concentration of benzocaine in the eluate was determined by spectrophotometric analysis at 283 mμ. The exact procedures employed determining the breakdown rates of the several systems are described in detail below.

Reagents.—*Benzocaine, U. S. P.*—Recrystallized from ethyl alcohol and water, m. p. 92–93°.

Caffeine, U. S. P.—Recrystallized from water, m. p. 235–237°.

Silicic Acid, Mallinckrodt chromatography grade was used to prepare the columns.

Diisopropyl Ether, that was washed once with 10% phosphoric acid and three times with distilled water. The phosphoric acid was used to remove any alkaline impurities present in the diisopropyl ether. The washing with water was done to remove excess acid and to saturate the diisopropyl ether with respect to water.

Apparatus.—Pyrex glass chromatographic columns 45 cm. long and 2 cm. in diameter. A tight fitting glass plunger to pack the columns. Beckman Model DU spectrophotometer. Constant temperature bath set at 30.0 ± 0.05°.

Chromatographic Columns.—The columns were packed according to the procedure of Higuchi and Patel (3). Distilled water was used as the internal phase and Skellysolve-C as the external phase to prepare the slurry.

PROCEDURE

Accurately weighed quantities of benzocaine or benzocaine and caffeine were placed into 500-cc. volumetric flasks. The reagents were dissolved in hot distilled water and brought up to a volume of approximately 400 cc. each. They were then allowed to cool down to 30° in a constant temperature bath.

To these, varying amounts of standard barium hydroxide solution, at 30° were added and the solution was brought up to 500 cc. with distilled water at 30°. The barium hydroxide solution was added to produce the desired normality with respect to OH^- concentration. A 20-cc. sample was taken from each flask for assay at this point. The remainder of the reaction solutions were placed in a constant temperature bath at 30° and at designated time intervals samples were removed for assay.

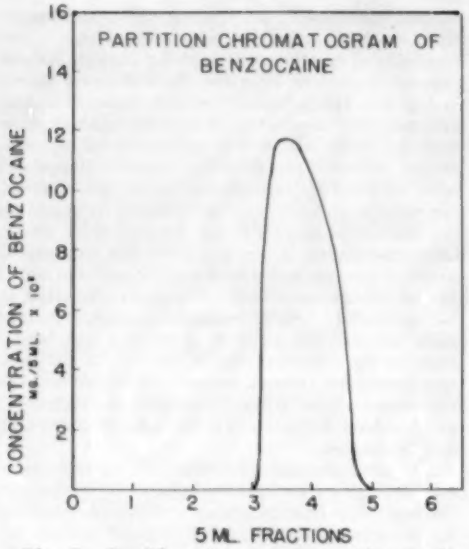


Fig. 5.—Partition chromatogram of benzocaine as eluted with diisopropyl ether from an aqueous solution of benzocaine and caffeine. The benzocaine was eluted from aqueous solution by the "slurry method" referred to in the text.

During the removal of a sample from a reaction flask, nitrogen gas was run over the mouth of the flask to prevent any carbon dioxide from the atmosphere from entering the flask and reacting with the barium hydroxide solution.

The 20-cc. sample removed from the reaction flask was placed into a 25-cc. volumetric flask to which was added 0.5 *N* acetic acid in 10% excess. The acetic acid neutralized any barium hydroxide remaining unreacted. The solution was then brought up to 25 cc. with distilled water. Exactly 5 cc. of the aqueous solution was placed on 5 Gm. of silicic acid in a 50-cc. beaker and mixed thoroughly. A slurry was made by adding 30 cc. of Skellysolve®-C. The slurry was then quantitatively transferred to the column, the beaker rinsed with 10 cc. of Skellysolve®-C, and the rinsings were added to the column. When removing the plunger, care was exercised not to remove any adhering slurry.

When approximately all the supernatant liquid had passed into the column, 1 cc. of diisopropyl ether was added and allowed to drain until almost all the supernatant liquid had again passed into the column. Then 1 cc. of diisopropyl ether was again added and allowed to drain as above. Following this step a 40-cc. portion of diisopropyl ether was added and the first 10 cc. was collected in a 10-cc. graduate and discarded. The next 25 cc. was collected in a 25-cc. volumetric flask for spectrophotometric analysis for benzocaine.

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The Stability of Ephedrine in Compound Ephedrine Spray N. F.*

By M. PERNAROWSKI and L. G. CHATTEN

Compound ephedrine spray N. F. IX shows a slight loss of ephedrine over a one-year period. The loss is not significant for this type of preparation and N. F. storage conditions may be considered as satisfactory. The quality of the materials used in the preparation of the oily spray must be carefully controlled to prevent an accelerated loss of ephedrine.

THE STABILITY of ephedrine in oily solution has been considered by several authors. The presence of peroxides in oily sprays was investigated by Lory, *et al.* (1). Jackerott (2) reported the formation of lipoid-soluble amides in oily ephedrine preparations and Welsh (3) described the reaction between ephedrine and benzaldehyde pointing out that the reaction product underwent some decomposition in oily preparations. The reaction of ephedrine with certain aldehydes is mentioned by Grant (4) and Davies (5). The former considered the possibility that some of these aldehydes resulted from the decomposition of the alkaloid which in turn reacted with these aldehydes to form loose compounds. Chatten and Pugsley (6) ascribed low recoveries of ephedrine from oily nasal sprays to inadequate mixing on the part of the manufacturer.

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The authors wish to thank Dr. L. I. Pugsley for his helpful suggestions. The assistance given by Dr. L. Levi in the preparation and interpretation of the infrared spectra of the mineral oils is greatly appreciated. Mr. M. Butler prepared the figures for publication.

The N. F. IX (7) states that compound ephedrine spray shall contain not less than 90% of the labeled amount of ephedrine. Of 22 commercial oily nasal sprays analyzed in this laboratory (Fig. 1), approximately 75 per cent failed to meet this lower limit. This large percentage of unsatisfactory preparations would not appear to be primarily due to inadequate mixing on the part of

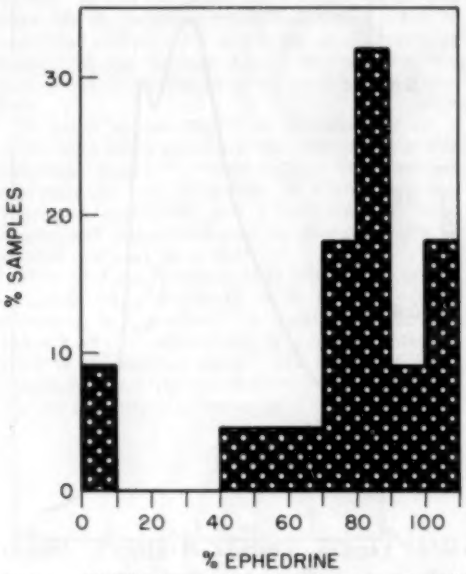

Fig. 1.—Distribution of the ephedrine content of 22 commercial oily nasal sprays.

the manufacturer. The problem of the stability of ephedrine in oily sprays may, therefore, be considered as more complex, involving several factors rather than one.

EXPERIMENTAL

In this study on the stability of ephedrine in mineral oil, storage of the finished product, manufacture of the oily spray, and quality of raw materials used in the manufacture were considered. The ephedrine and the mineral oil were checked for purity or stability. Other ingredients, however, may have some effect on the finished product but these were considered to be a constant in this study.

Storage Conditions of the Spray.—One thousand milliliters of compound ephedrine spray N. F. IX was prepared and analyzed by the nonaqueous method previously reported (8). This master solution was divided into five portions and stored as follows:

Sample I was stored in an open 200-ml. flask on a window sill.

Sample II was kept in a well stoppered 2000-ml. flask on the laboratory counter.

Sample III was stored in a well stoppered 200-ml. light resistant flask and kept in a dark, cool cupboard.

Sample IV was stored in a well stoppered 200-ml. flask and kept on the laboratory counter.

Sample V was stored in a well stoppered 200-ml. flask and kept in an incubator at 40°.

All samples were analyzed at monthly intervals.

In addition, the six colored oily nasal sprays that had been prepared and analyzed during the project on the nonaqueous determination of ephedrine in oily sprays (8) were stored in a dark cool cupboard and, at the end of one year, samples were removed and analyses conducted.

Method of Manufacture.—Compound ephedrine spray was prepared as specified in the N. F. IX with the exception of the water bath temperature. The ephedrine was solubilized at temperatures ranging from 40 to 90°.

Quality of Materials Used.—All samples of ephedrine were analyzed by A-P titration using chloroform as the solvent system.

Light Liquid Paraffin.—Three samples of light liquid paraffin were compared using the following procedures: (*a*) *Readily Carbonizable Substances.*—U. S. P. XIV procedure (9). (*b*) *Quality and Stability.*—The ultraviolet spectrophotometric procedure of Schnurmann, *et al.* (10), was used and spectra were obtained using a Cary recording spectrophotometer. (*c*) *Identification.*—Infrared spectra were obtained using a Perkin Elmer Model 21 recording spectrophotometer. A thin film of mineral oil was squeezed between two sodium chloride plates and the spectrum recorded throughout the 2.5 μ to 16 μ region. (*d*) *Aldehyde.*—Samples of light liquid paraffin were shaken with Schiff reagent to establish the presence of aldehydes. (*e*) *Break Down Time.*—The procedure followed was essentially that of Golden (11). (*f*) *Peroxide.*—The procedure followed was that of Chapman, *et al.* (12).

DISCUSSION

The results of the analysis of the stored samples of compound ephedrine spray N. F. IX over a period of one year are shown in Fig. 2.

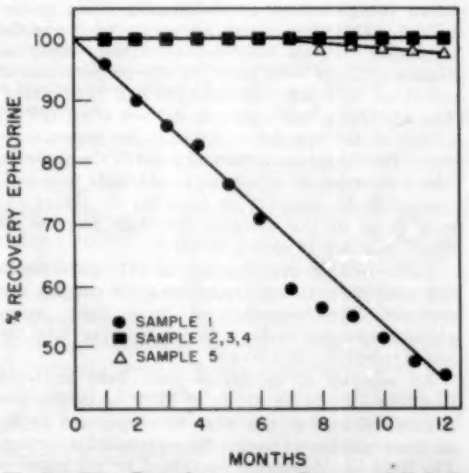

Fig. 2.—The effect of storage on Compound Ephedrine Spray N. F. IX.

The extreme storage conditions of *Sample I* were reflected in an approximate loss of 55% of the ephedrine content. A slight loss of ephedrine (2%) occurred in *Sample V*. It is unlikely that either of these storage conditions would occur in normal practice.

Samples II, III, and *IV* showed no significant loss of ephedrine over the one-year period although a trend downward appeared to have developed in *Sample II*. Calculation of the correlation coefficient showed that there was a difference in values over the one-year period. Percentagewise, this can be considered as insignificant since the change in recovery over a period of one year amounted to only 0.8%.

It would appear, therefore, that when N. F. IX storage conditions are adhered to, compound ephedrine spray shows no significant loss of ephedrine over a period of one year. This, however, was not confirmed when the colored samples of compound ephedrine spray were analyzed. These samples appeared to have lost approximately 5% of their ephedrine content in one year. Closer examination of the sprays showed that small, light brown globules had separated out from the solution. By use of gentle heat, these globules were solubilized and analysis now showed an almost theoretical recovery of the ephedrine content.

Samples of the spray were prepared by solubilizing the volatile ingredients at water bath temperatures ranging from 40 to 90°. Heating at the lower temperature for one hour showed no loss of ephedrine. When the volatile ingredients were heated at 90° for one hour, a loss of ephedrine of 3% was noted. This condition, however, is unrealistic since solution of these ingredients takes place in a little more than five minutes.

From these observations, it would appear that by following the N. F. monograph, there will be no significant loss of ephedrine during preparation or storage.

The separation of globules from the oily solution was evident in all samples prepared. However, no loss was found in *Samples II, III,* and *IV* since these were well mixed at monthly intervals and hence the globules were continually being resolubilized. This phenomenon appears to be due to the formation of an aldehyde-ephedrine complex. Similar globules were noted in oily preparations of ephedrine to which benzaldehyde had been added. The addition of the aldehyde did not affect the recovery of the ephedrine, although its presence reduced the sharpness of the end point in the analysis. The conversion of ephedrine to aldehyde to ephedrine-aldehyde complex as reported by Grant (4) may occur in this instance although there is no direct evidence of such a reaction.

Jackerott's (2) reported loss of 7% ephedrine in one year from an oily nasal spray is not too unrealistic. The formation of these light brown globules appears to be a factor entering into the loss of ephedrine on storage.

All samples of ephedrine used were analyzed and found to be of U. S. P. XIV quality. The mineral oil used in this work was subjected to the six tests mentioned under the experimental section. The light liquid paraffin contained no peroxides or aldehydes, met the U. S. P. XIV test for readily carbonizable substances and showed good stability by the tests of Schnurmann, *et al.* (10) and Golden (11). The ultraviolet spectrum of this oil is reported in Fig. 3. $E^{1\%}_{1cm.}$ was calculated to be 0.0182 and the ratio of the maximum absorption at 2,710 *A* to the minimum absorption at 2,490 *A* was found to be 3.5. Both factors indicate an oil of good quality and stability.

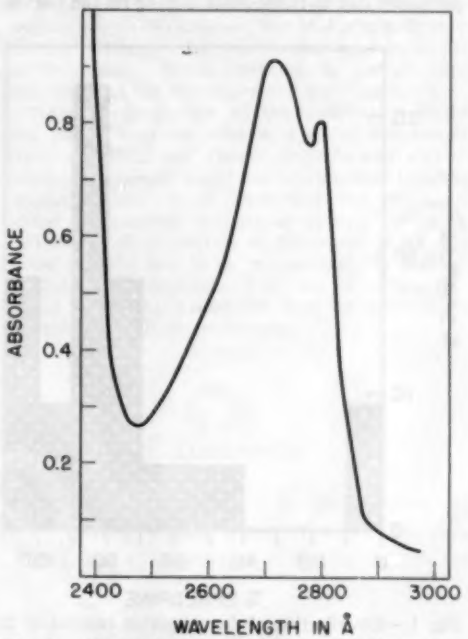

Fig. 3.—Absorption curve for light liquid paraffin.

In addition, two other samples of light liquid paraffin were tested by the above methods. One of these showed instability by the test of Golden (11) and was found to be high in aldehydes. The product, however, met the requirements of the three other tests. An ephedrine oily spray prepared from this oil was found to contain only 80% of the ephedrine content after one year of storage.

The infrared spectra of the three oils showed the characteristic absorption bands at 2,918, 2,861, 1,458, 1,378, and 720 cm.$^{-1}$ (13). A sample of U. S. P. XIV quality mineral oil which had been heated to 180° for two hours showed a change in spectrum. The absorption band at 2,861 was not as marked and the band at 720 was considerably broader.

The products of the oxidation of mineral oil have been reported as alkyl peroxides (1, 14), organic acids and esters (15), carbonyl-containing hydroxy acids and their anhydrides (16) and small amounts of unsaturated compounds (15). It would appear, therefore, that peroxides are first produced which in turn form aldehydes. The aldehyde content reaches a peak and then declines (17) in favor of organic and carbonyl-containing hydroxy acids which in turn form either esters or anhydrides.

Of all these decomposition products, peroxides and acid anhydrides would have the most effect on

the ephedrine. Lory, *et al.* (1) postulated that the peroxides acted as catalysts in the fixation of oxygen on ephedrine. They found that the peroxide effect was greater in vegetable oils than in mineral oils. This was substantiated in this laboratory by preparing an oily ephedrine spray using a vegetable oil base. The product showed a loss of 15% in sixty days.

Acid anhydrides may acetylate the ephedrine and hence make it unavailable for the A-P determination. Failure to isolate such an acetylated product from oily preparations which had shown a loss of ephedrine was probably due to the small quantities present in the samples used. Furthermore, the presence of an acetylated ephedrine is only a postulate.

It would appear that it is necessary to use a stable light liquid paraffin in the preparation of oily ephedrine sprays. A single stability test does not represent the over all quality of the mineral oil. Both van Hove (18) and Myddleton (19) have emphasized that several such stability tests must be applied to the oil under study.

It is perhaps fortunate that oily sprays of the compound ephedrine spray N. F. IX type are diminishing in importance. A number of manufacturers have discontinued this type of preparation in favor of the aqueous spray. The danger of lipoid pneumonia from the use of these oily preparations and the difficulty of adhering to N. F. storage conditions at the consumer level, will probably lead to the replacement of these preparations with aqueous types which show improvement in safety of use and adequacy of potency.

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The Application of Nonaqueous Titrimetry to the Determinations of Cholic, Desoxycholic, and Dehydrocholic Acids*

By R. CRISAFIO and L. G. CHATTEN

Cholic, desoxycholic, and dehydrocholic acids can be titrated visually in either benzene-methanol or N,N-dimethylformamide-chloroform as solvents, thymol blue as the indicator and either potassium methoxide solution or alcoholic potassium hydroxide as the titrant. Evidence is quoted to indicate that dissolving potassium hydroxide in methanol leads to the formation of potassium methoxide which will behave in a manner similar to the potassium methoxide formed by reacting metallic potassium with methanol.

WEAK ACIDS have been successfully titrated by application of nonaqueous procedures which would appear applicable to the determinations of cholic, desoxycholic, and dehydrocholic acids. The evidence also suggested that potassium methoxide solution and alcoholic potassium hydroxide might behave in a similar manner when used as titrants.

Fritz and Lisicki (1) cite the efforts of Folin and co-workers (2) who pointed out that many acids can be titrated accurately in benzene, toluene, chloroform, and carbon tetrachloride using sodium ethylate or amylate in the corresponding alcohol as titrant and phenolphthalein as the indicator. Fritz and Lisicki then describe their own success in titrating certain carboxylic acids both by potentiometric and visual techniques. Other more weakly acidic compounds were also successfully titrated. The titrant used was sodium methoxide in solution with benzene and methanol. The solvent, for the carboxylic acids was benzene-methanol, with thymol blue the indicator of choice.

* Received April 30, 1955, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.
The authors are indebted to Merck & Co. Inc., Rahway, N. J., for samples of dehydrocholic and desoxycholic acids.

Radell and Donahue (3) conducted potentiometric titrations on substituted fatty acids using sodium methoxide solution as titrant and benzene-methanol together with lithium chloride as the solvent solution. Fritz and Keen (4) used potassium methoxide solution to titrate phenols and because this titrant requires less methanol, they state it has advantages over sodium methoxide solution for the titration of phenols.

Clarke, Wooten, and Compton (5) used 0.05 per cent potassium hydroxide in butyl alcohol to titrate potentiometrically benzoic acid in butyl alcohol. Hall, Gibson, Phillips, and Critchfield (6) did a potentiometric titration on boric acid in glycerol and methanol using sodium hydroxide in methanol as the titrant. Deal and Wild (7) titrated potentiometrically a series of weak acids using either potassium hydroxide or tetrabutylammonium hydroxide in isopropyl alcohol as the titrants. The latter paper is also recommended for its useful list of references.

This study was undertaken to investigate a suitable procedure for the visual determination of cholic, desoxycholic, and dehydrocholic acids and to determine whether any practical difference resulted in the use of potassium hydroxide in methanol as titrant when compared with potassium methoxide solution, prepared by reacting metallic potassium with methanol and dissolving the reaction mixture in benzene-methanol as described (4). The N. F. IX method for determination of dehydrocholic acid in tablets and the nonaqueous procedures were also compared.

Apparatus.—Magnetic stirrer, buret graduated to 0.02 ml.

Reagents.—A. C. S. grade chemicals were used. Potassium methoxide solution 0.1 *N* was prepared as described (4) while the potassium hydroxide in methanol 0.1 *N* was prepared by dissolving potassium hydroxide in methanol and filtering. The indicator used was 0.5 per cent thymol blue in methanol.

Standardization.—Into 50 ml. of chloroform or 5 ml. methanol and 50 ml. benzene place two drops of thymol blue and neutralize. Add about 200 mg. of primary grade benzoic acid accurately weighed and titrate to a blue end point using the titrant of choice. Follow technique described under procedure.

PROCEDURE

Visual End Point Titrations.—(*a*) *Benzene-methanol Solvent System*—To 5 ml. of methanol and 50 ml. of benzene in a 150-ml. beaker add 2 drops of 0.5% thymol blue in methanol. Neutralize with a few drops of titrant. Add sample of bile acid and cover the beaker with a rubber dam. Place on a magnetic stirrer, push buret tip through small hole in the rubber dam and stir at slowest possible speed that will ensure mixing. Deliver titrant rapidly and titrate to a blue end point.

(*b*) *N,N-Dimethylformamide-Chloroform Solvent System*—Place sample of the bile acid in a beaker, add 2 ml. of dimethylformamide and stir with a rod until sample dissolves or is thoroughly wetted. Add 50 ml. chloroform and 2 drops of thymol blue. Titrate as indicated above but to a purple-blue end point. Perform a blank titration on the solvent system.

Dehydrocholic Acid Tablets.—Finely powder 20 tablets. Weigh accurately a quantity of powder equivalent to about 200 mg. of the acid. Using solvent system (*a*) place powder in a beaker, add methanol, mix well, and add benzene. Stir for fifteen minutes, add 2 drops of thymol blue and titrate to first bluish green color as the end point. Subtract blank.

Using solvent system (*b*), because of increased solubilizing capacity, it is possible to introduce a filtering technique to eliminate most of the excipients. Place the powdered sample in a small mortar. Thoroughly triturate with 2 ml. of dimethylformamide. Add about 25 ml. of chloroform, stir and pass through a small filter into the titration beaker. Remove the paper from the funnel and place into the mortar. Use 3 additional 15-ml. quantities of chloroform to wash thoroughly the paper and the mortar. Pass each washing through another filter into the titration beaker. Add 2 drops of thymol blue, cover with rubber dam, place on magnetic stirrer and titrate. Subtract blank.

Potentiometric Titrations.—A Beckman Model "G" *p*H meter, equipped with glass (No. 1190-80) and sleeve type calomel electrodes, was found satisfactory. As described in (1) and (3) 200 mg. of lithium chloride was dissolved in the benzene-methanol solvent to decrease resistance. If the electrodes are kept in water, prior soaking in benzene-methanol is not necessary, although this procedure is recommended (3). The titrations were carried out in an open beaker with hand stirring. The amount of CO_2 absorbed from the air in this procedure did not appear to interfere with quantitative recoveries.

RESULTS

Visual End Point Titrations.—Table I shows a comparison of the results obtained between the use of potassium methoxide solution and potassium hydroxide in methanol as titrants. Both reagents were prepared and standardized to be approximately 0.1 *N*. The solvent throughout was benzene-methanol and the indicator thymol blue. The data indicate that for the carboxylic acids concerned potassium methoxide solution has no advantage over the alcoholic potassium hydroxide solution while the latter has the advantage of being much simpler to prepare.

Visual titrations for these monocarboxylic acids in either methanol or 95% ethanol were not satisfactory. With thymol blue both solvents gave high blanks and indistinguishable shading was exhibited by the indicator. However, cholic acid could be

TABLE I.—A COMPARISON OF THE RESULTS OBTAINED USING TWO TITRANTS WITH BENZENE-METHANOL AS SOLVENT AND THYMOL BLUE AS THE INDICATOR

Bile Acid	Sample Size, mg.	% Recovery Pot. Methoxide Solution	Sample Size, mg.	% Recovery Pot. Hydroxide Methanol
Cholic	52.0	99.8	55.0	100.2
	51.8	99.4	49.6	100.4
	57.7	99.3	50.6	99.2
	164.0	99.1	154.0	99.5
	150.6	99.3	165.2	99.6
	156.0	100.3	167.0	99.6
Desoxycholic	51.2	99.6	50.2	100.0
	57.6	98.8	50.0	99.6
	50.2	99.2	52.4	99.6
	154.3	98.4	160.2	99.2
	149.0	98.8	142.2	100.2
	156.6	99.0	173.0	99.4
Dehydrocholic	53.2	100.5	59.6	101.3
	50.6	101.8	54.2	100.2
	51.6	101.3	64.6	101.6
	151.0	101.0	155.6	100.4
	168.8	100.6	140.4	100.6
	151.0	100.2	166.0	100.4

TABLE II.—RESULTS OBTAINED USING N,N-DIMETHYLFORMAMIDE-CHLOROFORM AS SOLVENT, THYMOL BLUB AS INDICATOR, AND POTASSIUM HYDROXIDE IN METHANOL AS THE TITRANT

Bile Acid	Sample Size, mg.	% Recovery
Cholic	99.7	99.2
	101.2	98.9
	105.6	99.1
Desoxycholic	104.2	100.4
	100.8	99.3
	160.6	99.3
Dehydrocholic	127.2	100.0
	124.2	100.3
	90.0	101.6

quantitatively determined in both these solvents using the potentiometric technique previously described.

Table II shows the results obtained using the dimethylformamide-chloroform mixture as the solvent. The end point is signaled by a change from yellow to a purplish-blue color and is pronounced, persisting long enough to be easily read. Some loss in color intensity may take place toward the end of a minute, but this does not preclude reproducible end points. Potassium hydroxide in methanol (0.1 *N*) was the titrant. These results indicate that this solvent system is as good as the benzene-methanol. The blank with thymol blue in this system is smaller than for benzene-methanol.

N. F. IX Procedure.—This procedure appears to be satisfactory for pure samples of dehydrocholic acid but reproducible results were obtained only with great difficulty for some of the commercial preparations. Some samples required stirring for periods up to one hour on the magnetic stirrer before solutions were effected in order to obtain quantitative recoveries; without stirring there was a marked tendency to obtain premature end points which become difficult to distinguish from the true end point. The addition of 100 ml. of water as prescribed, shortly before the end point is reached, may also lead to low results if added too soon. This is probably caused by some of the acid going out of solution. Table III shows a comparison of the recoveries on three commercial samples which have been assayed by three different methods. End points using Method A faded rapidly but could be detected with practice while the end points using Method B were sharper than A but recoveries were somewhat lower, possibly due to filtering. Results using Method C (N. F. IX) were obtained after prolonged periods of stirring. Single potentiometric determinations using potassium methoxide solution and benzene-methanol lithium chloride solvent showed recoveries of 98.4% for tablet 1, 105% for tablet 2, and 98.8% for tablet 3.

TABLE III.—COMPARISON OF THE PER CENT RECOVERIES FROM THREE COMMERCIAL SAMPLES OF DEHYDROCHOLIC ACID TABLETS USING THREE DIFFERENT PROCEDURES

Tablet	% Recovery Method A[a]	% Recovery Method B[b]	% Recovery Method C[c]
1	97.0	96.4	95.7
	98.3	96.2	95.7
	97.8	95.6	96.1
2	105.3	103.3	103.6
	104.5	102.5	101.8
	104.6	103.3	102.6
3	98.4	96.8	97.4
	98.1	97.2	98.0
	98.5	97.0	97.4

[a] Potassium methoxide solution titrant with benzene-methanol solvent and thymol blue.
[b] Potassium hydroxide in methanol titrant with N,N-dimethylformamide-chloroform solvent and thymol blue. Filtering technique used.
[c] N. F. IX procedure (aqueous NaOH titrant, alcohol-water solvent and phenolphthalein).

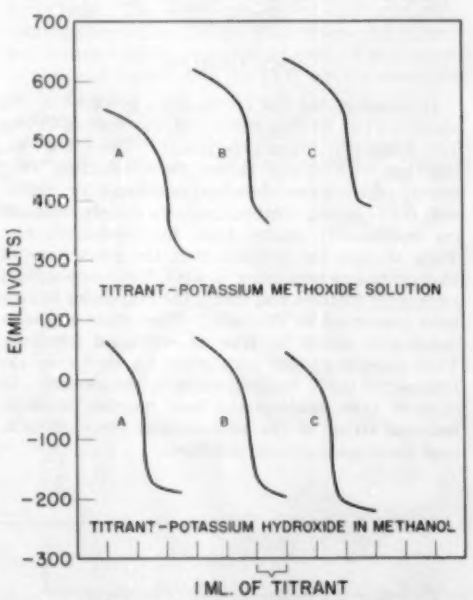


Fig. 1.—Titration of A-cholic acid, B-desoxycholic acid, C-dehydrocholic acid in benzene-methanol using glass-calomel electrodes.

It should be noted that presence of excipients makes the end points more difficult to interpret and in addition high results are obtained if acids such as stearic are present.

Potentiometric Titrations.—Figure 1 shows the curves obtained by titrating each of the bile acids using either potassium methoxide solution or potassium hydroxide in methanol as the titrants. These curves would indicate very little difference in the behavior of the titrant. Figure 2 shows the curves obtained titrating cholic acid in 95% ethanol and in methanol-lithium chloride with potassium hydroxide in methanol as the titrant. Recoveries in all instances were quantitative.

The glass-calomel electrode combination was satisfactory as was the glass-silver/silver chloride. Glass-antimony and glass-platinum were not found suitable.

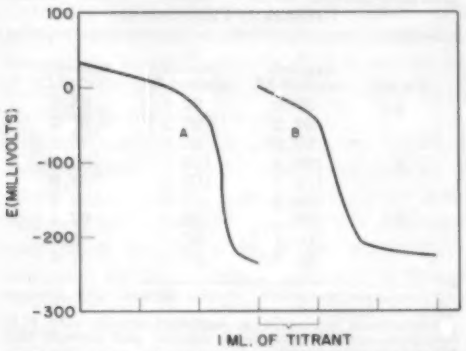
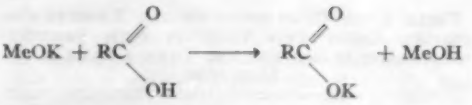


Fig. 2.—Titration of cholic acid in A-ethanol 95%, B-Methanol with lithium chloride using potassium hydroxide in methanol as the titrant.

DISCUSSION

In investigating the mechanisms involved in the use of the two titrants concerned, the work of Caldin and Long (8) appears pertinent. These authors, together with others before them, conclude that when hydroxides are dissolved in ethanol (or methanol) the resulting solution contains mainly ethoxide (or methoxide) rather than the hydroxide ion. They showed for instance that the percentage of hydroxide ions remaining in a 0.1 *N* ethanol-sodium hydroxide solution was 0.8%, the remainder having been converted to ethoxide. They state a similar conclusion would be true of methanol solutions. This percentage will vary from 1.5 to 4.1 in the presence of 0.2% to 1.0% water in the alcohol. In view of these findings the final reaction involved between either of the titrants and the carboxylic acid would appear to be as follows:



rather than a reaction involving the hydroxyl group as indicated by (7) in the instance of the alcoholic hydroxide. Any intermediate reactions would probably be governed by the type of solvent, since the carboxylic acids can be titrated in such media as methanol or chloroform (the small amounts of dimethylformamide were used to help solubilize the acid but are not necessary for the reaction to take place).

It would appear therefore that the more laborious practice of preparing potassium methoxide solution is not necessary in the titration of the carboxylic acids concerned. The alcoholic hydroxide was found to be an adequate titrant for this purpose. Whether diluting the alcoholic hydroxide with benzene to reduce the methanol content would be satisfactory in titrating weaker acids such as the phenols would bear investigation. The phenols were titrated successfully (4) using potassium methoxide solution, but other evidence (8) indicates that an alcohol hydroxide solution in benzene may be equally satisfactory.

SUMMARY

1. Cholic, desoxycholic, and dehydrocholic acids have been determined using potassium methoxide solution and potassium hydroxide in methanol as the titrants. Solvents used were benzene-methanol and N,N-dimethylformamide-chloroform with thymol blue as the indicator.

2. The N. F. IX method for assaying commercial samples of dehydrocholic acid tablets was found difficult to apply in order to obtain quantitative recoveries. Depending on the type of excipients used, two alternative methods were found to give results at least as satisfactory as the N. F. IX method and at the same time are much simpler to conduct.

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Determination of Sodium Carboxymethylcellulose*

By C. R. SZALKOWSKI and W. J. MADER

A method for the determination of sodium carboxymethylcellulose in antibiotic preparations. The copper salt of carboxymethylcellulose is precipitated and analyzed for carboxymethylcellulose colorimetrically using 2,7-naphthalenediol. Possible use of this method in quantitative determination of carboxymethylcellulose is suggested.

THE RAPIDLY INCREASING USE of sodium carboxymethylcellulose in pharmaceutical preparations has created interest in methods for its analysis. Methods which involve the precipitation of the copper or aluminum salts of carboxymethylcellulose (1, 2) and the determination of the metal content of the precipitate have been reported. The green color formed by the reaction of anthrone with carboxymethylcellulose has provided a basis for colorimetric methods (3–5). The specificity of the action of sulfuric acid to produce glycolic acid (6) from carboxymethylcellulose lends itself to a quantitative procedure of analysis in preparations containing materials which interfere with other colorimetric procedures. The use of 2,7-naphthalenediol both as a spot test (7) for glycolic acid and in a quantitative procedure (8) has been reported. Substances such as formaldehyde and glycolic acid will interfere, if present.

Attempts to apply a metal precipitation procedure and the various anthrone methods to antibiotic preparations were unsuccessful. Reproducible results were very difficult to obtain. The ultimate result of our investigation was a method consisting of a combination of the metal precipitation and colorimetric procedures.

EXPERIMENTAL

The procedure described was arrived at after study of all factors which have a bearing on the formation of the final color. This study included a comparison of the absorption curves of glycolic acid and sodium carboxymethylcellulose; effect of degree of substitution in the carboxymethylcellulose on the intensity of color; time required for color formation; calibration curves; and interfering substances.

Absorption Curves.—The absorption curves of the colors formed on reacting pure sodium carboxymethylcellulose and pure glycolic acid with a sulfuric acid solution of 2,7-naphthalenediol were determined in a Cary recording spectrophotometer. Figure 1 shows that both carboxymethylcellulose and glycolic acid have their maximum absorption at 530 mμ.

Effect of Time and Heat.—A correlation exists between the time required to hydrolyze the carboxymethylcellulose to glycolic acid and the intensity of the color formed. Samples of sodium carboxymethylcellulose were dissolved in diluted sulfuric acid and heated for definite periods of time. Then portions of the hydrolyzed solution were added to the naphthalenediol and heated for a period of thirty minutes and the resulting color read in a spectrophotometer at 530 mμ. The amount of color produced becomes constant after a two and a half hour hydrolysis. The same amounts of carboxymethylcellulose were added to the naphthalenediol reagent and heated for definite periods of time. The intensity of the color formed was constant after a three-hour heating period. This is shown in Fig. 2.

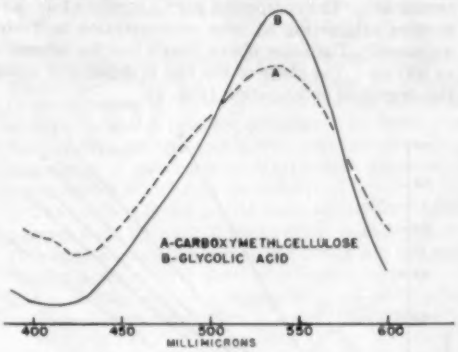

Fig. 1.—Absorption curves.

Stability of Color.—Sodium carboxymethylcellulose was treated with the naphthalenediol to develop the colored complex. The absorbance of the samples

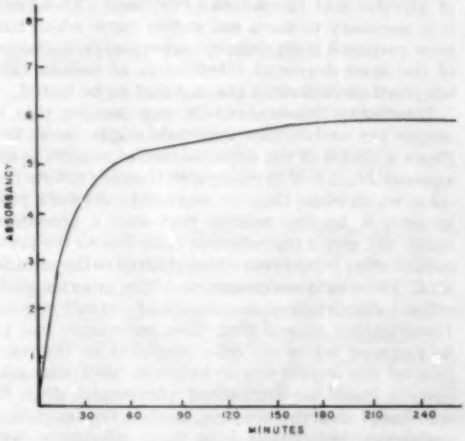

Fig. 2.—Effect of time of heating.

* Received October 22, 1954, from the Chemical Division, Merck & Co., Inc., Rahway, N. J.

of varying concentrations were determined at time intervals. The color, once formed, is stable for several hours and after the color is fully developed, practically any dilution volume can be used if it is desirable to reduce the sensitivity so that higher concentrations of carboxymethylcellulose can be determined.

Calibration Curves.—Samples of pure sodium carboxymethylcellulose of varying degrees of substitution were subjected to the test; concentrations of 20–300 μg. were used. Calibration curves were determined by plotting the absorbance against concentration. The compound gave a straight line calibration originating at zero concentration and absorbance. The color obeys Beer's law for amounts to 300 μg. The slope of the line is dependent upon the degree of substitution (Fig. 3).

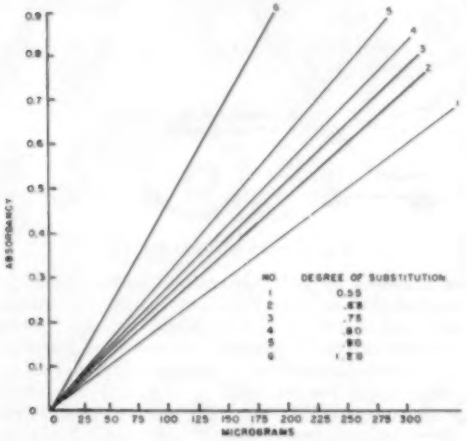

Fig. 3.—Calibration curves.

Effect of Degree of Substitution.—It was observed from the calibration curves that different samples of sodium carboxymethylcellulose produce different amounts of color. Therefore, the degree of substitution of each sample was determined. The greater the degree of substitution, the greater amount of glycolic acid formed on hydrolysis. Therefore, it is necessary to use a calibration curve which has been prepared from sodium carboxymethylcellulose of the same degree of substitution as sodium carboxymethylcellulose in the material to be tested.

Interfering Substances.—It was thought that a simple yet satisfactory approach might lie in the direct addition of the naphthalenediol reagent to an aqueous solution of the sample and heating to form the color which could then be measured. As work progressed, it became evident that such a procedure would not give a reproducible color due to the presence of other ingredients which charred in the sulfuric acid. These substances were penicillin, procaine penicillin, dihydrostreptomycin, and streptomycin. Investigation showed that these substances had to be removed before the color produced by the reaction of the carboxymethylcellulose with the acid reagent could be determined photometrically. It was found that the simplest method for separating carboxymethylcellulose from these substances was the precipitation procedure.

The result of our investigation is the procedure consisting of introducing a dilute copper sulfate into a well agitated aqueous solution of the sample under acid conditions (*p*H 2.5); adjusting the *p*H to 4.1 with dilute ammonia to obtain complete precipitation of the copper carboxymethylcellulose; filtering and washing the precipitate; dissolving the washed precipitate in diluted sulfuric acid; hydrolysis of the carboxymethylcellulose to glycolic acid; and finally the colorimetric determination of the glycolic acid formed.

Reagents.—*Hydrochloric Acid.*—A 10% solution; *Ammonium Hydroxide.*—A 3% solution; *Copper Sulfate.*—A 1% aqueous solution of $CuSO_4 \cdot 5H_2O$. *Diluted Sulfuric Acid.*—A mixture of 500 ml. of water and 300 ml. of concentrated sulfuric acid; *Naphthalenediol Reagent.*—Dissolve 100 mg. of 2,7-naphthalenediol (Eastman) in 200 ml. of concentrated sulfuric acid and allow the resulting solution to stand for at least eighteen hours in the dark; *Sodium Carboxymethylcellulose Standard.*—To prepare the standard it is necessary to use sodium carboxymethylcellulose of the same degree of substitution as the sodium carboxymethylcellulose in the sample to be analyzed. Weigh 100.0 mg. pure sodium carboxymethylcellulose into a 200-ml. volumetric flask, dissolve in 100 ml. of water, carefully add 6.0 ml. of concentrated sulfuric acid, mix and cool to room temperature. Dilute to mark with diluted sulfuric acid and mix thoroughly. Each milliliter contains 0.50 mg. of sodium carboxymethylcellulose.

METHOD

Preparation of Sample.—Weigh an amount of the sample to be tested corresponding to 2 to 4 mg. of sodium carboxymethylcellulose into a 250-ml. beaker. Add 50 ml. of water and introduce a mechanical stirrer with a glass paddle and shaft and the electrodes of a *p*H meter. Stir until solution is complete. Adjust the *p*H of the solution to 2.5 with dilute hydrochloric acid, add 50 ml. of cupric sulfate solution and stir to mix thoroughly. Adjust the *p*H of the mixture to 4.1 by dropwise addition of ammonium hydroxide.

Let the mixture stand for one hour to allow the precipitate to settle. Decant as much of the supernatant liquid as possible through a sintered-glass filter with the aid of suction. Transfer the precipitate to the filter with the aid of a fine stream of water and wash with several 10-ml. portions of water.

Place the filter into a Fisher filtrator containing a 50-ml. volumetric flask. Carefully add 5-ml. portions of the diluted sulfuric acid (heated to about 60°) to the filter and gently triturate with a glass rod, apply suction and draw the solution through each time. About five treatments are usually sufficient to dissolve and transfer the precipitate to the volumetric flask. Cool the solution to room temperature and make up to mark with diluted sulfuric acid.

Measure 2-ml. aliquots of the acid solution into 2.5 x 20 cm. glass-stoppered tubes (the length of the tube is an important factor in preventing water vapors from diluting the sulfuric acid during the reaction in the boiling-water bath). Add from a pipet or buret 20 ml. of naphthalenediol reagent to each

TABLE I.—RECOVERY OF SODIUM CARBOXYMETHYLCELLULOSE

Composition	No. Detd.	% Recovery	% S. D.
Pen[a]-NaCit[b]-CMC[c]	4	98.5	±1.5
PP[d]-NaCit-Pen-CMC	4	101.5	±1.9
DH[e]-Pen-NaCit-CMC	4	100.4	±1.6
DH-Pen-St[f]-NaCit-CMC	4	102.2	±2.0
PP-Pen-DH-NaCit-CMC	4	101.5	±1.5
PP-Pen-DH-NaCit-CMC	4	100.3	±1.2
DH-PP-ST-NaCit-CMC	4	100.8	±2.0
DH-PP-ST-Pen-NaCit-CMC	4	101.6	±2.3
PP-Pen-ST-NaCit-CMC	4	102.2	±2.5

[a] Pen—Penicillin G potassium.
[b] NaCit—Sodium citrate.
[c] CMC—Sodium carboxymethylcellulose.
[d] PP—Procaine penicillin.
[e] DH—Dihydrostreptomycin sulfate.
[f] ST—Streptomycin sulfate.

tube. Stopper the tubes and make the mixture homogeneous by carefully shaking the tubes. Place the stoppered tubes in a boiling water bath for three hours. Remove the tubes from the bath and cool them in an ice bath for five minutes, then add from a pipet or buret 20 ml. of diluted sulfuric acid. Mix thoroughly and let stand for thirty minutes. Read the color in a spectrophotometer set at 530 mμ against a blank prepared at the same time from the reagents.

Preparation of Standards.—Measure 5 and 10 ml. of the standard sodium carboxymethylcellulose solution into separate 50-ml. volumetric flasks and dilute to mark with diluted sulfuric acid and mix well. Pipet 2-ml. aliquots into glass-stoppered tubes, add 20 ml. of naphthalenediol reagent, stopper and mix well. Place in boiling water bath and proceed as with the sample.

DISCUSSION

The data resulting from the application of the method to nine preparations are given in Table I. An average recovery of 98.5% or better was obtained.

Although the method was developed primarily for sodium carboxymethylcellulose in antibiotic mixtures, because of its specificity it can be adapted to other mixtures. The specificity of the action of sulfuric acid to produce glycolic acid from carboxymethylcellulose makes this possible. Previous knowledge of the degree of substitution, however, is necessary in calculating the results.

The method is not particularly suited for routine control work because of its length. It is estimated that approximately six to eight hours are required to run a set of four determinations. However, it has a definite use as a referee method or in other analytical work where time is not a critical factor.

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Composition of the Volatile Oil from *Chamaecyparis lawsoniana* Stump Heartwood*

By GENE KRITCHEVSKY and ARTHUR B. ANDERSON

The oil contained approximately 5% *d*-α-pinene, 3% *d*-limonene, *p*-cymene (trace), 3% terpinolene, 0.5% *d*-camphor, 6% *l*-α-fenchyl alcohol, 2.3% *d*-bornyl acetate, 11% *d*-borneol, 27.5% *d*-α-terpineol, 4% *l*-citronellol, 3.5% *l*-citronellyl acetate, 4% *l*-bisabolene, white solid m. p. 129.7–131.0° (trace), 7% tricyclic sesquiterpene, 15% *d*-canadene (6), white solid m. p. 134.5–135.5° (trace), 4% *l*-cadinol, and 0.5% *l*-cadinyl acetate.

CHAMAECYPARIS LAWSONIANA (Port Orford cedar) occupies a rather limited range along the western coastal area of southern Oregon and northern California. In addition to its application in the normal wood using channels, an essential oil was commercially available for a time produced from sawdust and mill residues. The oil yields from this source averaged about 1.6 per cent (9). Apparently the processing of this material solely for the purpose of oil recovery in these yields, proved to be uneconomical.

Higher yields of volatile oil would be expected from the stump heartwood. One of us (ABA) made a preliminary examination several years ago of the yields of steam volatile oil obtainable

* Received March 5, 1955, from the Forest Products Laboratory, University of California, Richmond, Calif.
The authors are indebted to W. Ruhman of the Wood Chemicals Company, Coos Bay, Ore., for the sample of oil used in this investigation.

from old Port Orford cedar stumps. A large sampling of stumps from Coos Bay, Oregon, indicated that this material would yield from 4 to 9 per cent volatile oil. This investigation deals with the chemical analysis of the steam volatile oil obtained from a composite sampling of stump heartwood.

In 1914, Schorger (6) received a steam distilled oil from selected resinous pieces of Port Orford cedar. The yield was reported to be 10 per cent. This oil was stored in an amber bottle for four years after which it was analyzed. The oil was treated with 10 per cent sodium carbonate and the basic solution steam distilled. This rectified oil was reported to contain 60–61 per cent *d*-α-pinene, 6–7 per cent dipentene, 11 per cent borneol, 11.5 per cent ester as a bornyl acetate, and 6–7 per cent cadinene. The esters were present as formate, acetate, and caprate; each of these acids also being present in the free state.

In 1927, Thurber and Roll (9) analyzed an oil obtained in 1.6 per cent yield by steam distillation of Port Orford cedar sawdust. This oil was reported to contain 46 per cent *d*-α-pinene,

TABLE I.

Fraction	Boiling Range ° C./13 mm.	n_D^{25}	d^{25}	α^{25}	% Distilled	% Oil	Weight
1	44.2–45.0	1.4643	0.8544	+46.6	4.3	4.3	57.42
2	45.0–54.4	1.4652	0.8493	+53.2	4.9	0.6	8.5
3	54.4–60.7	1.4680	0.8491	+65.2	5.6	0.7	9.1
4	60.7–62.4	1.4699	0.8442	+80.4	7.1	1.5	20.6
5	62.4–65.0	1.4722	0.8460	+77.8	7.6	0.5	7.1
6	65.0–68.7	1.4760	0.8582	+52.0	8.2	0.6	7.9
7	68.7–71.6	1.4816	0.8688	+21.6	9.0	0.8	10.8
8	71.6–74.9	1.4838	0.8798	+11.6	9.8	0.8	11.3
9	74.9–81.5	1.4794	0.9058	+12.4	10.2	0.4	5.7
10	81.5–84.2	1.4742	0.9337	+ 8.0	10.9	0.7	9.5
11	84.2–85.6	1.4732	0.9474	+ 4.0	12.5	1.6	21.9
12	85.6–86.9	1.4732	0.9495	+ 2.4	14.2	1.7	22.5
13	86.9–87.0	1.4732	0.9495	+ 3.0	14.6	0.4	5.8
14	87.0–88.4	1.4732	0.9495	+ 4.8	16.1	1.5	19.3
15	88.4–89.6	1.4734	0.9474	+ 7.4	17.4	1.3	17.2
16	89.6–91.9	1.4741	0.9459	+10.4	18.2	0.8	10.9
17	91.9–95.0	1.4754	0.9430	+16.6	19.4	1.2	16.1
18	95.0–96.7	1.4767	0.9430	+19.4	19.9	0.5	7.2
19	96.7–99.3	1.4771	0.9421	+24.8	20.9	1.0	13.0
20	99.3–100.8	1.4797	0.9406	+52.4	30.7	9.8	130.4
21	100.8–101.5	1.4804	0.9426	+63.4	34.7	4.0	52.8
22	101.5–101.7	1.4802	0.9399	+68.6	37.3	2.6	34.2
23	(98.8)–100.3	1.4793	0.9399	+54.6	37.9	0.6	7.9
24	100.3–101.4	1.4797	0.9392	+64.4	39.4	1.5	19.8
25	101.4–102.0	1.4797	0.9378	+66.6	41.3	1.9	25.5
26	102.0–102.2	1.4797	0.9366	+70.0	46.6	5.3	70.0
27	102.2–103.3	1.4794	0.9361	+70.4	52.0	5.4	71.8
28	103.3–103.9	1.4794	0.9373	+65.2	54.1	2.1	27.5
29	103.9–104.4	1.4796	0.9373	+59.0	55.7	1.6	20.6
30	104.4–105.9	1.4784	0.9382	+50.8	56.6	0.8	11.6
31	105.9–108.3	1.4758	0.9207	+37.2	57.9	1.3	16.8
32	108.3–109.5	1.4712	0.9092	+22.6	58.6	0.7	9.3
33	109.5–111.0	1.4667	0.8875	+10.6	60.0	1.4	19.0
34	111.0–111.9	1.4629	0.8803	+ 6.6	62.0	2.0	26.0
35	111.9–112.9	1.4620	0.8916	+12.4	63.3	1.3	17.0
36	112.9–114.7	1.4620	0.9029	+17.4	63.8	0.5	7.2
37	114.7–116.2	1.4624	0.9075	+ 7.2	65.0	1.2	15.9
38	116.2–118.2	1.4682	0.8995	− 4.0	66.5	1.5	19.7
39	118.2–119.5	1.4771	0.8928	− 8.2	67.2	0.7	9.0
40	119.5–121.5	1.4835	0.8935	− 8.0	67.9	0.7	9.8
41	121.5–125.5	1.4905	0.9010	− 4.6	68.5	0.6	7.5
42	125.5–129.9	1.4957	0.9120	− 4.8	69.0	0.5	6.8
43	129.9–131.2	1.4998	0.9164	− 3.2	70.0	1.0	13.6
44	131.2–133.2	1.5019	0.9176	0.0	70.9	0.9	12.3
45	133,2–134.5	1.5054	0.9144	+ 2.4	72.4	1.5	19.4
46	134.5–135.7	1.5050	0.9116	+ 3.0	73.6	1.2	16.4
47	135.7–136.6	1.5059	0.9122	+14.4	76.5	2.9	39.0
48	136.6–137.3	1.5060	0.9128	+17.8	78.7	2.2	29.7
49	137.3–138.0	1.5073	0.9128	+61.4	86.2	7.5	99.1
50	94.0–94.4/1.1 mm.	1.5088	0.9156	+82.0	91.4	5.2	69.6
51	94.4–114.4	1.5097	0.9498	+26.8	92.6	1.2	16.6
52	114.4–117.8	1.5077	...	−35.6	94.5	1.9	24.9
53	117.8–120.8	1.5080	...	−41.0	95.3	0.8	11.8
54	Column hold up	1.5118	...	...	96.4	1.1	14.8
	Residue	...	...	...	98.1	1.7	23.2

3 per cent *d*-limonene, 26 per cent *d*-borneol, 21 per cent *d*-cadinene, and 4 per cent *l*-cadinol.

EXPERIMENTAL

The oil analyzed in this investigation was steam distilled from stump wood obtained from the Coos Bay, Oregon, area. The stump heartwood was reduced to fine chips and steam distilled from a 50-gal. steel still using a copper coil condenser. The decanted oil, which was obtained in 5% yield, was stored in tin-lined containers and was analyzed four and one-half years later. After drying over sodium sulfate, the oil had the following properties: n_D^{23} 1.4880; d^{23} 0.9309; $[\alpha]_D^{23}$ + 29.7°; free acid, as acetic acid 0.11%; ester as bornyl acetate, 6.28%; free alcohol, as borneol 34.6%; total alcohol as borneol, 40.0%.

The fractionation of 1338 Gm. of oil was effected by 90-cm. Todd column of 25 mm. diameter, packed with glass helices. The oil was fractionated at 13 mm., using reflux ratio of 10:1. The physical properties of the various fractions obtained are reported in Table I.

Crystallization of *d*-borneol in fraction 20 necessitated a lower reflux ratio as well as use of external heat. After collection of fraction 49, the pressure was lowered to 1.1 mm.

The characteristic odor of *p*-cymene was observed in fractions 7 and 8, but the compound was not isolated in reasonably pure condition. Its presence is probably due to decomposition of terpinolene.

The physical constants recorded for fractions 11 and 12 are those of the filtrate after removal of the 1-α-fenchyl alcohol. Similarly, the constants of fraction 20 apply to the filtrate obtained by removal of the borneol.

Sesquiterpene fractions 40–50 were tested for azulenogenic compounds by treatment of two drops of each fraction with 1 ml. glacial acetic acid and a little bromine vapor. A positive test was indicated by the presence of a crimson color, turning gradually to dark blue. A maximum color intensity was observed in fractions 43–45. Since no azulenes could be isolated through dehydrogenation with palladium-charcoal, it is presumed that the precursor of the color is present in very small concentration.

The crystalline cadinol of fractions 50–54 is of particular interest since it corresponds closely to a compound isolated by Plattner and Markus (5). Structural work on this compound is in progress in connection with our work on the extractives of *Chamaecyparis lawsoniana*.

The two white crystalline solids obtained in trace amounts, m. p. 129.7–131.0° and 134.5–135.5°, are probably di- and trihydroxy-*p*-menthanes, respectively.

The various components were identified as follows:

d-α-Pinene.—Fraction 1 was converted by $KMnO_4$ oxidation to pinonic acid, m. p. 70.0–70.8° (1).

d-Limonene.—Bromination of fraction 4 yielded limonene tetrabromide, m. p. 105.0–105.5° (4).

p-Cymene.—Potassium permanganate oxidation of fractions 7 and 8 gave a very small amount of terphthalic acid (10).

Terpinolene.—Fraction 8 was brominated and the recrystallized derivative melted at 116.0–117.0°. The melting point was not depressed when mixed with an authentic sample of terpinolene tetrabromide.

l-α-Fenchyl Alcohol.—The crystalline material from fractions 11 and 12 melted at 47.0–48.0° and $[\alpha]_D^{23}$ − 9.8° (95% ethanol). The *p*-nitrobenzoate was prepared and after recrystallization had a m. p. of 107.0–107.7° and $[\alpha]_D^{25}$ − 19.8°, literature value −20.0° (carbon bisulfide) (3).

d-Camphor.—Fractions 13–15 were combined with the filtrates from fractions 11 and 12. After cooling and filtering to remove *l*-α-fenchyl alcohol, the filtrate was fractionated. The 2,4-nitrophenylhydrazone derivative (2) was prepared from the fraction boiling at 86.3–86.9°/13 mm. Recrystallization from alcohol gave a derivative with m. p. 175.5–176.5°. There was no change on mixed melting point with an authentic sample of *d*-camphor 2,4-dinitrophenylhydrazone.

d-Borneol.—Filtration of fraction 20 gave 13.5 Gm. of crude borneol, which on recrystallization had a m. p. of 207.8–209.3°; $[\alpha]_D^{25}$ + 38.5° (benzene). The *p*-nitrobenzoate melted at 136.0–136.7°, which was not depressed on admixture with an authentic sample.

d-α-Terpineol.—Fractions 21–28 were refrigerated and then filtered to give 62 Gm. of white crystals, m. p. 32.4°. An additional quantity of crystals was recovered from the filtrate. The recrystallized product had a m. p. of 37.4–38.3°; $[\alpha]_D^{23}$ + 105° (benzene). The *p*-nitrobenzoate melted at 138.5–139.0°, while the nitrosochloride melted at 106.5–107.5°.

d-Bornyl Acetate.—Fractions 20–29 contained 8% ester calculated as bornyl acetate. The ester was purified via the butyl borate method (7). A fraction with n_D^{25} 1.4665; d^{25} 0.9400, and $[\alpha]_D^{25}$ + 29°, was saponified with alcoholic KOH. The acid component proved to be acetic acid, since the *p*-bromophenacyl derivative gave no change in melting point with an authentic derivative. The alcohol recovered was identified as *d*-borneol through the preparation of its *p*-nitrobenzoate derivative.

l-Citronellol.—Purification of fraction 34 through its borate ester yielded an alcohol, n_D^{25} 1.4621, d^{25} 0.8748, and $[\alpha]_D^{23}$ −2.1°. Titration with bromine indicated the presence of one double bond. The silver salt of the acid phthalate was prepared and was crystallized from benzene-methanol, m. p. 124.8–125.6°. The allophanate derivative was also prepared, by mixing 3 Gm. of the alcohol, 5 Gm. potassium cyanate and 10 Gm. of glacial acetic acid. After this mixture was allowed to stand four hours with occasional shaking, it was diluted with 100 ml. of water, refrigerated for twelve hours, filtered and the solid dried on a clay plate. The product upon several recrystallizations from ethyl ether had a m. p. of 107.9–108.3° and gave no change in melting point with an authentic sample of *l*-citronellol allophanate.

l-Citronellyl Acetate.—Fractions 34, 37, and 39 contained 21, 47, and 28%, respectively, of citronellyl acetate according to their saponification equivalents. The ester was purified by the butyl borate method, then saponified with alcoholic potassium hydroxide. The acidic portion yielded *p*-bromophenacyl acetate, m. p. 84.7–85.4°. The alcohol was identified through its allophanate, m. p. 108.0–108.4°.

l-Bisabolene.—Fraction 40 was distilled over sodium b_{25} 137°, n_D^{25} 1.4920, d^{25} 0.870, and $[\alpha]_D^{25}$ − 10.5°. Refractionation gave a product with $[\alpha]_D^{25}$ − 14.8°. The hydrochloride derivative was prepared which had a melting point of 76.6–78.6°.

Tricyclic Sesquiterpene.—Sulfur dehydrogenation of fraction 44 gave cadalene, identified as the picrate, m. p. 113.5–114.0°. No change on mixed melting point with an authentic sample. Treatment of fraction 44 with dry hydrogen chloride in ether gave a small amount of cadinene hydrochloride, m. p. 118.0–118.8; $[\alpha]_D^{25}$ − 36.7° (chloroform) which crystallized only after two weeks of refrigeration.

Fractions 43, 44, and 45 were combined and distilled over sodium. The center cut, d^{25} 0.9193, was dehydrogenated with 5% palladium on charcoal. Three grams of the oil and 0.1 Gm. catalyst were heated at 215° for six and one-half hours, yielding 2.1 Gm. of trinitrobenzene adduct, m. p. 112.1–113.3°. Since the *d*-canadene, fractions 49 and 50, yielded the same results under identical conditions on dehydrogenation, but widely different results in hydrochloride formation, it seems that fractions 43–45 represent a distinctly different sesquiterpene. Hydrogenation of the redistilled oil indicated 1.25 double bonds. It seems that this compound is a tricyclic sesquiterpene.

d-Canadene (8).—Fractions 49 and 50 were combined and distilled over sodium. The palladium dehydrogenated product formed a trinitrobenzene adduct with m. p. 111.8–112.9°. This sesquiterpene readily formed a dihydrochloride with m. p. 118.5–119.0°, and $[\alpha]_D^{25}$ − 37.0°. This corresponds closely to the hydrochloride of *l*-cadinene.

l-Cadinol.—Analysis of fraction 52 indicated a sesquiterpene alcohol.

Anal.—Calcd. for $C_{15}H_{26}O$: C, 81.02; H, 11.34. Found: C, 81.39; H, 11.67.

The *p*-nitrobenzoate derivative was prepared and after two crystallizations from methanol, it melted at 137.2–137.9°. The ester was saponified, yielding a crystalline product with m. p. 74.8–75.4° and $[\alpha]_D^{25}$ − 38.5°. The *l*-cadinol isolated by Plattner and Markus from Java citronella oil melted at 72.5°, with $[\alpha]_D$ − 39.2° and its *p*-nitrobenzoate had a m. p. of 136°.

Fractions 52 and 54 gave good yields of cadinene dihydrochloride when allowed to stand two weeks with ethyl ether saturated with hydrogen chloride.

Cadinyl Acetate.—Saponification of a composite sample of fractions 51–54, indicated the presence of 0.5% ester, calculated as cadinol acetate.

$C_{10}H_{18}O_2$: Fractions 39 and 40 when cooled, yielded about 100 mg. of a white solid, m. p. 129.7–131.0° upon crystallization from benzene.

Anal.—Calcd. for $C_{10}H_{18}O_2$: C, 70.79; H, 10.57. Found: C, 70.55; H, 10.66.

$C_{10}H_{18}O_3$: Fraction 49, when diluted with petroleum ether, yielded approximately 100 mg. of a white solid which upon recrystallization from benzene had a m. p. of 134.5–135.5°.

Anal.—Calcd. for $C_{10}H_{18}O_3$: C, 64.29; H, 9.77. Found: C, 64.49; H, 9.74.

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A Suggested Improvement in the U. S. P. Method for Determining Morphine in Opium*

By HAROLD W. BRICKLEY and F. A. WHIPPLE

This suggested modification of the officially described U. S. P. assay method for opium has been found to reduce the required over-all assay time by at least one third. The efficiency of extraction appears to be practically the same by both methods. The modified method should eliminate any errors that may be due to the prolonged filtration that is now required by the U. S. P. procedure. The accuracy of this proposed method has been found to be at least equal to that of the currently official procedure and the results obtained by both methods are in good agreement.

IN AN EFFORT to shorten the U. S. P. assay procedure for opium gum, a study has been undertaken to determine how this could best be accomplished without affecting the accuracy of this long established but relatively tedious official method. Since the filtering and evaporation processes are the most time-consuming operations in the method, this was considered to be the logical area of investigation. It was felt that the addition of some inert material would effectively facilitate the dispersion of the opium gum in the warm water extractions and thereby speed up the subsequent filtrations.

The dispersing agents tried in this investigation

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were magnesium carbonate and tribasic calcium phosphate, both of which are officially recognized as diluents for powdered opium in the U. S. P. XIV. Magnesium carbonate proved to be of little value and it also produced low results in the assay. On the other hand, results obtained by the use of tribasic calcium phosphate were found to be quite satisfactory. The filtration time was further reduced by utilizing vacuum filtration in preference to the gravity method.

Recoveries of morphine from six different lots of opium gum assayed by the U. S. P. method and by our modified method are recorded in the following table.

TABLE I.

Sample No.	U. S. P. XIV Method (% of Morphine Found)	Modified Method (% of Morphine Found)
1	12.97–12.81	12.71–12.74
2	10.61–10.65	11.03–10.88
3	11.90–11.94	12.60–12.60
4	10.12–10.22	10.33–10.19
5	13.52–13.49	12.91–12.98
6	13.01–12.94	12.70–12.54

ASSAY PROCEDURE

Weigh accurately 6 grams of opium which, if fresh, should be in very small pieces and, if dry, in a fine powder. Place the opium in a 250-ml. glass-stoppered flask and add 1.5 Gm. of tribasic calcium phosphate followed by 30 ml. of distilled water. Place the flask on a steam bath and disperse the opium with the aid of a stout glass rod. Stopper the flask and shake the mixture occasionally during a ten-minute heating period. Filter the hot contents of the flask by vacuum filtration into a 500-ml. suction flask using a three-inch Buchner funnel and No. 1 filter paper. Allow the suction to continue until most of the water has been removed, and the material on the filter is fairly dry. Carefully remove the precipitate from the filter paper in the Buchner funnel by peeling it off with a spatula and returning it to the original flask. Add 30 ml. of distilled water and repeat the heating and shaking treatment, filtering the contents of the flask through the same filter into the same 500-ml. suction flask. (If the filter paper should tear while the residue is being removed from it, the residue and torn filter both may be returned to the extraction flask and again extracted. A new filter paper is then used in the Buchner funnel.) Carry out this washing operation two more times and add the aqueous extractions to those in the original suction flask. Now evaporate the combined aqueous extracts in the 500-ml. suction flask by heating on the steam bath and passing a strong current of air directly into the flask. (Note —The evaporation procedure may actually be started after completing the first filtration.) When the volume is reduced to about 35 Gm., add three grams of calcium hydroxide. Mix well and continue the evaporation until the liquid is reduced to about 25 Gm. Now completely transfer the mixture to a 125-ml. tared, glass-stoppered flask with the aid of small portions of distilled water and add distilled water until a weight of 54 Gm. is obtained. Close the flask and mix thoroughly. Filter with the aid of vacuum through a three-inch Buchner funnel fitted with a No. 1 filter paper, into a dry 250-ml. suction flask.

Place exactly 34 Gm. of the filtrate corresponding to 4 Gm. of opium into a tared 125-ml. glass-stoppered Erlenmeyer flask, add 2 ml. alcohol, 15 ml. of ether and after mixing thoroughly, add one gram of ammonium chloride. Stopper the flask, shake it frequently during ten minutes and set aside overnight at a temperature of 5–10°. Decant and filter the ether layer through a medium sintered-glass filtering funnel using a vacuum. Rinse the flask and contents with 15 ml. of ether, again decant the ether layer and pass through the filter. Now pour the water layer and morphine crystals upon the filter and continue the filtering operation until all of the liquid has passed through. Rinse the flask with small portions of water saturated with morphine and filter these through the funnel, using a total of 40 ml. of the morphinated water. Remove the vacuum and place 1 ml. of cold water upon the morphine crystals in the funnel, then apply the vacuum again until all the water has drained and discard all the filtered liquids.

Rinse the stopper of the flask with 20 ml. of boiling reagent methanol, collecting the methanol in the flask which still contains a few of the morphine crystals. Pour the boiling methanol upon the morphine crystals in the funnel and filter into a clean dry 250-ml. suction flask. Repeat this operation three more times or until all of the morphine has dissolved. Add 25 ml. of 0.1 *N* H_2SO_4, 50 ml. of distilled water and evaporate on a steam bath to a volume of about 50 ml., passing a current of air into the flask to facilitate evaporation.

Cool and titrate the excess acid with 0.1 *N* NaOH using methyl red T. S. as the indicator.

Each milliliter of 0.1 *N* H_2SO_4 is equivalent to 28.53 mg. of anhydrous morphine.

Properties and Applications of Powdered Polysaccharide Acids III*

Inhibition of Microbial Growth by Carboxymethylcellulose

By JOSEPH V. SWINTOSKY† and ALFRED M. KAUFMAN

Oxygen uptake studies have been conducted with *M. pyogens* var. *aureus*, *E. coli*, *S. faecalis*, and *C. albicans* in media buffered with carboxymethylcellulose. Under these conditions the rate appears to be related to *p*H in a restricted range. Oxygen uptake of *C. albicans* was not greatly influenced in the range *p*H 3.9–5.1; however, a high degree of oxygen uptake inhibition was demonstrated for the bacteria at the lower end of this *p*H range. Since carboxymethylcellulose shows a high buffering capacity at about *p*H 4, and possesses physical properties which lend themselves to pharmaceutical compounding, these studies suggest some possible applications in the treatment of skin and epithelial tissues.

WE HAVE OBSERVED that the rate of oxygen uptake of *M. pyogenes* var. *aureus*, *E. coli*, *S. faecalis*, and *C. albicans*[1] is related to the presence in the culture medium of variable amounts of the free acid form of carboxymethylcellulose (HCMC). Since HCMC has only recently been prepared (1, 3) and because it possesses a number of unique properties as an acid which might make it a useful therapeutic agent (1, 2), these studies were initiated to evaluate its potentialities.

APPARATUS AND MATERIALS

Apparatus.—A Lardy Circular Warburg-type respirometer with Warburg manometers was employed for measuring oxygen uptake (4, 5, 6). A model G Beckman *p*H meter was employed for *p*H measurements.

Culture Media and Reagents.—Sterile solutions and suspensions in distilled water were prepared as follows:

Solution N	*% Composition*
D (+) dextrose	2.50
Potassium phosphate, monobasic	0.01
Ammonium phosphate, dibasic	0.01
Magnesium sulfate	0.01
Sodium chloride	0.50

Solution S: This was identical to Solution N except that a 1% HCMC solution prepared by ion exchange (1) was used as the vehicle in place of distilled water.

Solution P: This was identical to Solution N except for the addition of 2% HCMC powder.

Bouillon Broth (7, 8, 9)	*% Composition*
Beef extract	0.50
Bacto peptone	0.70
Sodium chloride	0.50
Isotonic Dextrose Medium	
D (+) dextrose	5.39
Sabouraud's Maltose Agar[2] *(9)*	
Bacto nutrient agar, dehydrated	2.00
Bacto peptone	1.00
Maltose	4.00
Candida Culture Medium[2]	
Bacto peptone	1.00
D (+) dextrose	2.00
Sodium chloride	0.50
Beef extract	0.30

EXPERIMENTAL PROCEDURE

Preparation of the culture.—*M. Pyogenes* var. *aureus* was grown on a Bacto nutrient agar plate for twenty to twenty-four hours at 38° (7, 8), then washed off the plate with bouillon broth into a sterile flask. This was diluted further with sterile bouillon broth until the suspension transmitted 10% of the light transmitted by the initial broth under the same conditions, as determined with a Cenco colorimeter using a blue filter. Such a culture suspension is designated as possessing a density of 10. All culture preparations were adjusted to this density.

S. faecalis was prepared in the same way as *M. pyogenes* with the exception of temperature which was 45° (7).

The preparation of *E. coli* was identical to *M. pyogenes*, but transfer was made to isotonic dextrose medium (8–11).

C. albicans was grown on a Sabouraud's maltose agar plate for twenty to twenty-four hours at 38° (12, 13), then transferred to the sterile candida nutrient medium.

Determination of oxygen uptake.—Two cc. of culture suspension was added to each Warburg flask. The addition of 1 cc. solution N with either solutions S or P provided combinations of 4 to 6 flasks each containing 3 cc. of single culture suspensions (Table I) varying in *p*H from about 3.5–5. Similar culture suspensions were prepared at this point for original *p*H determinations.

* Received March 22, 1955, from the School of Pharmacy, University of Wisconsin.

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This paper is based in part on a thesis submitted by Alfred M. Kaufman to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Master of Science.

[1] The *S. faecalis* culture was obtained from Dr. C. W. Seastone, Professor of Medical Microbiology at the University of Wisconsin. The other cultures were obtained from Dr. W. B. Sarles, Professor of Agricultural Bacteriology at the University of Wisconsin.

[2] Filtration preceded autoclaving.

TABLE I.—THE COMPOSITION OF FLASK SUSPENSIONS IN A TYPICAL RUN GIVING *p*H VALUES FOR A FOUR-HOUR TIME INTERVAL

Flask No.	*E. coli*[a] Suspension, cc.	Soln. N, cc.	Soln. P, cc.	Initial *p*H	Final *p*H	Average *p*H
1	2	0.95	0.05	4.93	4.71	4.82
2	2	0.90	0.10	4.42	4.35	4.38
3	2	0.85	0.15	4.14	4.10	4.12
4	2	0.80	0.20	3.97	3.99	3.98
5	2	0.50	0.50	3.40	3.40	3.40
6	2	0.00	1.00	3.02	3.00	3.01

[a] At 38°.

Agitation of the flasks at constant temperature[2] proceeded for one hour prior to balancing the manometers. Then after the lapse of another hour readings were taken every fifteen minutes for two consecutive hours; and *p*H of the microbial suspensions was redetermined at the conclusion.

RESULTS AND DISCUSSION

Table I describes the compositions of flask suspensions of *E. coli* during one oxygen uptake experiment, and illustrates the relative constancy of *p*H observed during all of these studies.

Figure 1 illustrates the oxygen uptake observed for *E. coli* during a two-hour interval when the flask suspensions correspond in composition to that given in Table 1. The data yield a family of essentially straight lines indicating that the rate of oxygen uptake was relatively constant for each suspension over this time interval. The slopes of the respective lines decrease with decreasing *p*H, and at *p*H 3.01 it is observed that oxygen uptake was negligible.

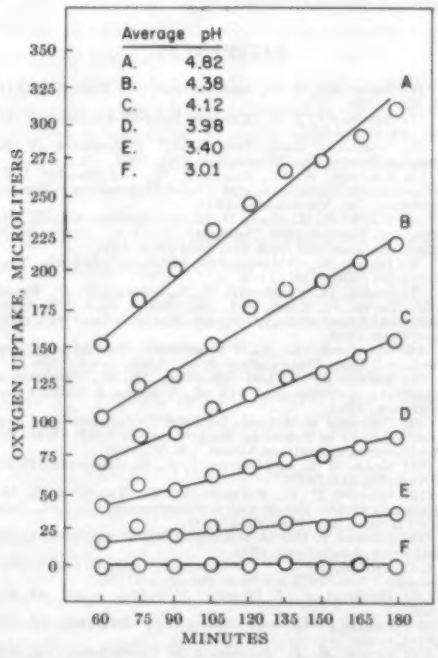

Fig. 1.—Typical oxygen uptake data for *E. coli* at 38° C. where suspended HCMC powder was employed to create media of constant *p*H.

That the data for *E. coli* are typical of the results derived from the four microorganisms is exemplified by Fig. 2, which relates the log rate of oxygen uptake to the average *p*H of the microbial suspensions. The plots are linear under the conditions of the study; and summarize the pertinent data relating the oxygen uptake of each microbial suspension to the *p*H induced by the HCMC inhibitor.

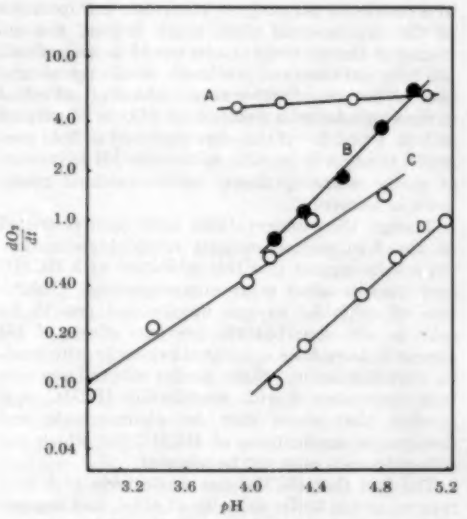

Fig. 2.—Graph relating log rate of oxygen uptake to *p*H of culture suspensions. (A), *C. albicans;* (B) *M. pyogenes* var. *aureus;* (C), *E. coli;* (D), *S. faecalis.*

This would suggest that a colloidal aqueous solution or suspension of HCMC is an effective inhibitor of the oxygen uptake of the bacteria studied, particularly when the concentration is sufficient to effect a *p*H near or less than four. It is much less effective in *C. albicans* as molds and yeasts are generally less sensitive to acidic environments than most bacteria.

The microbial inhibition observed is due principally perhaps to a *p*H effect since the composition of the media employed varied only with respect to HCMC concentration. Yet the possibility of HCMC anions or undissociated molecules acting as inhibitors cannot be excluded completely. Nonetheless, regardless of the mechanisms involved, the microbial oxygen uptake can be related positively to the *p*H.

The straight line plots of Fig. 2 would suggest that the oxygen uptake of the microorganisms may be influenced by the following relationships:

$$\frac{dO_2}{dt} = 10K \cdot pH + C \qquad (1)$$

[2] The temperature was 38° in all instances except with *S. faecalis* where the temperature was 45°.

or in the logarithmic form

$$\log \frac{dO_2}{dt} = K \cdot pH + C \qquad (2)$$

The latter is an equation of a straight line, and defines the plots of Fig. 2. The slopes of these plots correspond to K; and K may be considered a *p*H sensitivity constant singularly characteristic for each of the microorganisms under the conditions of the experiments. The K values for the bacteria varied between 0.7 and 1, whereas the K value for *C. albicans* was about 0.08. A low value of K would indicate a relative nonsensitivity to *p*H, while a high value of K would denote relatively high *p*H dependency. By the very nature of the biochemical reactions it describes, this equation must be valid only in a restricted *p*H range. Therefore, extrapolation of the experimental plots much beyond the extremes of the observed results would be unjustified. We have not observed previously such a representation of the quantitative relationships of microbial oxygen uptake as a function of *p*H, as illustrated in Eqs. 1 and 2. If this were observed to hold generally, it might be possible to describe *p*H sensitivity of many microorganisms, under standard conditions, in terms of K.

Though these observations have been restricted to the four microorganisms under investigation, the results suggest that this inhibition with HCMC may likewise affect other microorganisms. Inhibition of microbial oxygen uptake and growth by acids is not new; but the previous effects of *p*H appear to have been associated primarily with readily diffusible acids. Since similar effects have now been demonstrated with nondiffusible HCMC, it is possible that there may be pharmaceutic and therapeutic applications of HCMC for which the diffusible acids may not be adapted.

The fact that HCMC has a relatively high acid reserve, ample buffer capacity at *p*H 4, and appears to be relatively nonirritant, nondigestible and nonabsorbable makes it unique. These properties may be useful in therapeutics where a persistent residual acidic environment is desired. When spray dried, HCMC is a fine nongritty white powder. As such, it could be used in jellies, creams, lotions, suspensions, ointments, powders, tablets, and suppositories. Specifically, it may prove valuable in the treatment of certain types of vaginitis, where an abnormally high *p*H generally prevails. Since the normal reaction of the vagina is usually about *p*H 4, the use of HCMC might be rationalized to bring about the normal *p*H in order to reestablish the normal bacterial flora when they have been suppressed by the overgrowth of pathogens favored by the altered *p*H relationships. The utility of HCMC for this purpose might be augmented by using it in combinations with antiseptics, fatty acids, antibiotics, and other chemotherapeutic agents (14–17). Similarly, HCMC may have highly desirable effects in the treatment of certain skin and epithelial infections. Preliminary studies have shown it to possess marked spermicidal activity. In such a product HCMC could serve as an effective ingredient.

SUMMARY

1. Rate of oxygen uptake was studied manometrically in cultures of *E. coli*, *S. faecalis*, *M. pyogenes* var. *aureus*, and *C. albicans* suspended in HCMC giving a *p*H range of about 3.5–5.

2. Rate of uptake was suppressed by HCMC, and appears to be related to *p*H according to the following equation:

$$\log \frac{dO_2}{dt} = K \cdot pH + C$$

When rate of oxygen uptake is expressed in microliters per minute, the K values for the bacteria varied from 0.7 to 1. The value K may be interpreted as a *p*H sensitivity constant characteristic of each organism under the conditions of the study. Sensitivity of *C. albicans* was of a low order.

3. HCMC may have pharmacologic advantages, either alone or in conjunction with other agents, in the treatment of skin, epithelial or vaginal infections where acidic *p*H levels are desirable because of its high acid reserve, ample buffering capacity at *p*H 4, apparent nontoxicity, nondiffusibility, and nonabsorbable character. Preliminary studies have shown it to possess marked spermicidal activity.

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Paper Chromatography of Several Veratrum Alkaloids*

By JOSEPH LEVINE and HENRY FISCHBACH

Veratrum alkaloids may be sharply separated by paper chromatography on paper which is distinctly moist with pH 3.5 buffer. The location of the alkaloids is detected with the indicator dye bromphenol blue, which is sensitive to 1 μg. of alkaloid.

A NUMBER of reports describing the characterization of alkaloids by paper chromatography have been published. In general, these involve resolving the alkaloids with water-saturated organic solvents. After development, the position of the alkaloid is usually detected with a general alkaloidal reagent, such as iodine, potassium iodoplatinate, or Dragendorff's reagent. In a procedure for separating a large number of alkaloids, Curry and Powell (1) report a single R_f value for veratrine alkaloids as a group. The chromatography of several individual veratrum alkaloids has been reported (2), in which detection is accomplished by immersing the paper in a tray of concentrated sulfuric acid. These chromatograms require approximately 250 μg. of alkaloid; the R_f values increase with increasing quantities of alkaloid.

The present paper describes a chromatographic separation of the veratrum alkaloids encompassing a new detection reagent, which produces permanent spots and which is sensitive to less than 1 μg. of alkaloid; and a pretreatment of the paper which results in the production of dense, discrete spots, free from tailing. Ascending chromatography is used. Development is rapid; a movement of 10 cm. requires one to one and one-half hours, and no lengthy preequilibration of the paper is required. R_f values are independent of the quantity of alkaloid. Two resolving systems are used; one for rapid-moving, the other for slow-moving, alkaloids.

DETECTION REAGENT

The position of the alkaloids on the developed chromatogram is detected by means of the indicator dye, bromphenol blue (tetrabromophenol sulfonphthalein). This has been previously used for the detection of acids on paper chromatograms (3). The majority of the alkaloids, including those derived from veratrum, are sufficiently alkaline to convert the dye to the blue salt modification. The feebly basic alkaloids, such as caffeine, cannot be detected with this reagent.

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The alkaloids are located by spraying the paper with a solution of the dye in a volatile solvent, followed by humidification of the paper. Since the formation of the blue color results from the ionic reaction between the basic alkaloid and the acid form of the indicator dye, humidification is essential in order to furnish sufficient moisture to provide an ionization medium on the paper.

The behavior of the background was found to vary in different batches of chromatographic paper. In some batches the background remained yellow, allowing the blue spots at the position of the alkaloids to stand out; in other batches, however, the background turned blue gradually, or, with some samples of paper, quite rapidly, engulfing the alkaloid spots. This is prevented by pretreatment of the paper with pH 3.5 buffer; paper so treated retains the yellow background permanently.

PREPARATION OF PAPER

The general practice in paper chromatography is to equilibrate the paper prior to development with the vapors of the developing solvent or of the equilibrium mixture in those cases where the solvent is saturated with water, although the need for this diminishes as the volume of the chromatographic chamber is decreased (4). In the chromatography of alkaloids, the paper is often impregnated with an electrolyte, either a neutral salt or a buffer, prior to such equilibration. In the treatment of the veratrum alkaloids on paper prepared by these procedures we obtained a certain degree of resolution; however, the spots were elongated, with considerable tailing. Mixtures of alkaloids appeared as streaks with nodes at the points of highest concentration.

In order to obtain discrete separation, it is necessary that the paper have a considerably higher moisture content than that produced by simple equilibration. Block, *et al.*, (5) state that hydration of the paper results in more uniform chromatograms and very compact spots. Sabatino (6) sprays his paper with sodium carbonate solution "to achieve uniform dampness" prior to development. Pechet (7) impregnates filter paper with water or the saturated aqueous phase of the solvent mixture for chromatography of very polar steroids.

The proper moisture content is achieved by thoroughly drenching the paper with water or aqueous buffer, and removing the excess moisture by blotting between sheets of paper toweling. The solution of the alkaloid is spotted and the paper allowed to air-dry to the point where the halos, resulting from evaporation of the solvent, fade out. This operation takes two to five minutes. The paper at this point must remain definitely moist; if it dries to the point where the extreme limpness of the moist paper begins to give way to the stiffness or crispness of dry paper, the resulting chromatogram will exhibit streaks, rather than develop the

discrete spots obtained on the moist paper. The degree of wetness attained by storing the paper in a jar over water even for one week was insufficient to achieve the condition for optimum resolution. Although Curry and Powell (1) noted variations in R_f values of alkaloids with degree of dryness of the paper, we have found the R_f values to be quite constant; however, in order to guard against variations resulting from differences in solvent batch, paper, temperature, etc., a known sample is run on each paper to serve as reference point. In order to maintain reproducible conditions, the paper should be introduced into the chromatographic chamber when it reaches a definite stage of wetness. When too much water is left on the paper, the solvent front will be diffuse and quite irregular, as contrasted with the straight line obtained under proper moisture conditions. Although no measurable index is available to govern the moisture requirements, the procedure as described, together with the feel of the paper which is acquired with a little experience, permits the attainment of the desired conditions.

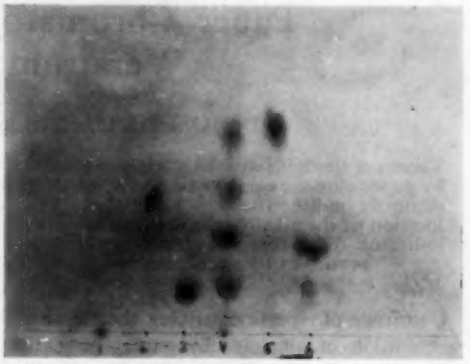

Fig. 1.—Position 1—germine; 2—jervine, 3—neoprotoveratrine (protoveratrine B), 4—mixture of five alkaloids, 5—veratramine, 6—protoveratrine A. The chromatogram shows that this material contains a small amount of neoprotoveratrine.

RESOLVING SYSTEMS

In the development of the moist chromatogram, the use of solvents having a very low mutual solubility with water, such as benzene, tends to produce a diffuse solvent front. Butyl acetate acidified with formic acid, with increasing amounts of butanol added for alkaloids with slower migration rates, was found to be very satisfactory.

The migration sequence of the different alkaloids remains unchanged while the magnitude of the R_f value increases with increasing butanol content of the solvent. Hence, considerable latitude is available in choice of solvent for optimum separation of selected groups of alkaloids. Similarly, acetic acid provides the same pattern as formic acid, but at lower R_f values. The solvent should be freshly prepared, since changes in R_f values result with old solvent, presumably due to esterification during standing.

For the greater number of veratrum alkaloids, the solvent system *n*-butyl acetate : *n*-butanol : formic acid (25:5:1 by volume) is used. Either the 85–90% or the 98–99% grade of formic acid may be used. The resultant R_f values with this solvent are: protoveratrine A, 0.3; neoprotoveratrine (protoveratrine B), 0.12; jervine, 0.45; veratramine, 0.65; germitrine, 0.3; neogermitrine, 0.25 (a mixture of the latter two is separated); veratridine, 0.03; germine, 0.0. These values were obtained at a room temperature of approximately 25°. Figure 1 shows the pattern given by germine, protoveratrine A, neoprotoveratrine, jervine, and veratramine, and a mixture of all five.[1] Approximately 5 μg. of each alkaloid was used both in the individual spots and in the mixture. In spotting the mixture, one drop (approximately 2 μl.) of solution of each alkaloid was applied to the same spot, with drying between successive additions.

For the slower moving alkaloids, the solvent is prepared by adding 1 ml. of formic acid to the separated solvent layer of the system *n*-butyl acetate:*n*-butanol:water (10:25:10 ml.). With this system, the R_f values of veratridine and germine are 0.3 and 0.08, respectively. This solvent system is suitable also for other alkaloids such as quinine, strychnine, codeine, etc. The alkaloids may be spotted on the paper as the free alkaloid or the salt without effect on the R_f value.

It was not found possible with any modification of solvent system to separate protoveratrine A and germitrine. For identification purposes, however, it was found that partial methanolysis of these alkaloids results in mixtures of alkaloids giving markedly different patterns. Protoveratrine yields spots at R_f 0.3, 0.2, 0.1, and 0.03, while germitrine yields principal spots at R_f 0.3 and 0.25. On longer standing, strong spots appear at R_f 0.03.

EXPERIMENTAL PROCEDURE

The chromatographic chamber can consist of any simple airtight container, rectangular or cylindrical. In this laboratory glass-stoppered cylindrical museum jars, 11½ inches high and 2½ inches in diameter, are used. Sheets of Whatman No. 1 filter paper for chromatography, 8 x 8 inches, are used. These are fashioned into cylinders held together at the top by a staple or a paper clip, and rested directly on the floor of the jar, immersed in a layer of about ¼ inch of resolving solvent. To prepare the paper, draw the base line one inch from the bottom, and on it mark spots to locate the positions for the introduction of the sample. Draw a line 10 cm. above this to fix a definite distance for the migration of the solvent front. Wet the paper by pouring over it approximately 10 ml. of McIlvaine's phosphate-citrate buffer, *p*H 3.5 (8). After short draining, sandwich the paper between sheets of paper toweling; rub the surface of the toweling for some seconds to remove as much moisture as will be absorbed, and repeat with fresh paper towels.

By means of a glass tube drawn out to a capillary tip, add a small drop (*ca.* 2μl.) of a solution of the alkaloid in chloroform or alcohol (1 to 10 mg. per ml.). Evaporation of the solvent will leave a white halo. After several minutes, as the paper continues

[1] The authors are indebted to the Research Laboratories of Riker Laboratories, Inc., and of E. R. Squibb and Sons for furnishing the alkaloids, which were used directly as obtained; and to J. F. Nicholson, Division of Microbiology, Food and Drug Administration, for preparation of the photograph.

to dry, the halo will fade into the background. Note the feel of the paper. It should feel definitely moist and limp, but not so wet as to transfer water to the fingers. At this point, place the paper in the chromatographic chamber.

When the solvent front reaches the 10-cm. mark, remove the paper and allow to air-dry or dry in a ventilated 100° oven. Spray the paper with a solution of approximately 10 mg. bromphenol blue per 100 ml. of chloroform or other volatile solvent. After spraying, suspend the paper over an open steam bath and direct a vigorous flow of steam onto the paper; do not allow to get too wet, or the dye will dissolve and run. As moisture is absorbed, blue spots will appear against a yellow background at the location of the alkaloid. The spots will remain visible for at least a period of several months.

For partial methanolysis of the ester alkaloids, dissolve several mg. of the alkaloid in 0.5 ml. $CHCl_3$. Add 0.5 ml. 80% methanol; introduce several particles of 20-mesh carborundum to serve as boiling stones, and heat in a beaker of water at 65–70° for fifteen minutes. Apply the resultant solution as is to the paper.

SUMMARY

A procedure is described for the separation of the veratrum alkaloids by paper chromatography. The use of the indicator dye, bromphenol blue, as detection reagent for alkaloids is introduced. It is shown that, for the solvent systems used, moistening of the paper beyond the extent attainable by simple equilibration is essential to achieve suitable resolution.

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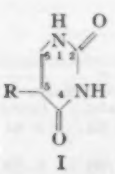
The Synthesis of Uracils as Anticonvulsants*

By J. H. BURCKHALTER and HOMER C. SCARBOROUGH†

A convenient laboratory synthesis has been devised by which a series of new 5-alkyluracils has been prepared for evaluation as anticonvulsants. Certain other 5-substituted uracils and their N,N-dimethyl derivatives, and related compounds, have also been synthesized. The anticonvulsant activity of uracil and thymine has been surpassed by several members of this new series.

AS A RESULT of the observation that both uracil (I) (R = H) and thymine (R = CH_3) possess significant anticonvulsant activity (1), we decided to prepare certain related alkyl and aryl uracils for pharmacological evaluation. Since thymine was observed to afford better protection against electrically induced convulsions in mice than either uracil or 6-methyluracil (1) a group of uracils to be substituted in the 5-position received our first attention (I).



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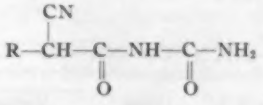

II

* Received May 5, 1955 from the Laboratory of Pharmaceutical Chemistry, University of Kansas School of Pharmacy, Lawrence.

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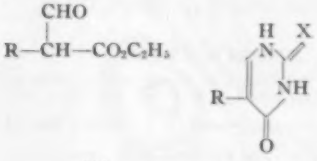
5-Alkyluracils have been prepared by a number of different procedures (2). One of the most promising appeared to be through the one step procedure of Rupe, which has been utilized for the preparation of both uracil and thymine (3). This method involves the partial catalytic reduction, hydrolysis and subsequent ring closure in an aqueous medium of α-cyanoureides (II). While a small amount of impure 5-butyluracil was obtained when 50% alcohol was used as a solvent, the procedure became impractical when R was larger than methyl because of the insolubility of the intermediates (II). Nevertheless, the intermediate α-cyanoureides themselves were found to have interesting anticonvulsant activity.

The α-cyanoureides (II) of Table I were prepared, following the procedure of Johnson and Bergman (3), from the corresponding α-cyano acids which were prepared by saponification of the esters obtained by either the direct alkylation of the sodium salt of ethyl cyanoacetate with alkyl halide (4), in the case of the α-cyanobutyrate and α-cyanocaproate, or by the reductive alkylation of ethyl cyanoacetate with propionaldehyde (5), in the case of the α-cyanovalerate.

Most of the 5-substituted uracils of Table II were prepared by the condensation of an α-formyl ester (III) with either thiourea or urea, by modification of the procedure of Anderson and

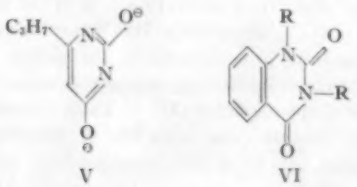
co-workers (6). The substituted 2-thiouracils (IVa) obtained by this reaction were then hydrolyzed by means of chloroacetic acid to substituted uracils (IVb) following the general procedure of Johnson and Hemingway (7). The direct synthesis of the uracils from urea was used in only two cases because of the extremely poor yields.



III IV

The 1,3-dimethyluracils of Table II were prepared through the treatment of the alkyl- or aryluracil with alkaline dimethyl sulfate, following the technique which Davidson and Baudisch used to prepare 1,3-dimethyluracil (8). Table III lists the comparative physical properties of certain uracils and their N-substituted derivatives, which we have prepared.

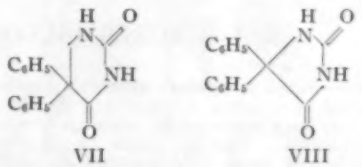
Treatment of 6-propyluracil with alkaline diethyl sulfate resulted in the introduction of only one ethyl group as evidenced by elementary analysis and by solubility characteristics. The alkylated product is only slightly soluble in cold water, but it dissolves readily in 5% aqueous sodium hydroxide. Assignment of the ethyl group to the one position is based on the work of Hilbert and Johnson (9) who determined that the N_1 position was alkylated over the N_3 position in the case of 2,4-dimethoxyuracil (2,4-dimethoxypyrimidine); in alkaline solution 6-propyluracil would be expected to exist in an enol form, V, comparable to 2,4-dimethoxypyrimidine.



V VI

Table II also contains 6-propyluracil and 6-phenyluracil and their N,N-dimethyl derivatives. 6-Propyluracil was obtained by hydrolysis of commercially available 6-propyl-2-thiouracil, while 6-phenyluracil and 1,3-dimethyl-6-phenyluracil have been described earlier by Johnson and Hemingway (7).

For pharmacological study, it was also of interest to prepare the known 2,4(1,3)-quinazolinedione and its dimethylated derivative, 1,3-dimethyl-2,4(1,3)-quinazolinedione (VI), because, it can readily be seen, these compounds may be considered to be benzouracils. The former was synthesized from anthranilic acid and potassium cyanate according to the method of Bogert and Scatchard (10). Dimethylation to give the latter compound could be effected in only small amount by the procedure of Clark and Wagner (11) because of the low solubility of the starting material in alkali. However, larger amounts were conveniently prepared by the action of dimethyl sulfate upon the crude sodium salt of the intermediate as it was isolated directly from its preparative medium (10).



VII VIII

5,5-Diphenylhydrouracil, (VII), may be thought of as a homolog of the drug diphenylhydantoin (VIII) in which a methylene group has been inserted between positions 1 and 5. It was synthesized by the reaction of ethyl α,α-diphenyl-β-aminopropionate hydrochloride with potassium cyanate. The preparation of the free basic ester has earlier been described by Bockmühl and Ehrhart (12), while Burtner and Cusic have reported the synthesis of α,α-diphenyl-β-aminopropionic acid and its *methyl* ester hydrochloride (13). The type of ring closure used for VII is similar to that used by Posner for the preparation of 5,6-diphenyl, 6-ethyl-6-phenyl

TABLE I.—α-CYANOUREIDES, R—CHCN—CO—NH—CO—NH2

Compound, R,	Cyanoester, Lit. ref.	m. p. °C.	Yield,* %	Recryst. Solvent	Ureide, Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
C_2H_5	4	176.5–178	26	Water	$C_6H_9N_3O_2$	46.34	46.20	5.84	5.84
n-C_3H_7	5	179.5–180	34	Water-methanol	$C_7H_{11}N_3O_2$	49.69	50.38	6.56	6.59
n-C_4H_9	4	182–183	32	Water-acetone	$C_8H_{13}N_3O_2$	52.45	52.49	7.15	7.33

* Cyanoester to ureide.

TABLE II.—URACILS

Uracil	Prep. Method	Yield, %	m. p., °C.	Recryst. Solvent	Molecular Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
Uracil	B[a, b]	97	339–341[c]	Water					
1,3-Dimethyl	C	97	120–121[e]	Ethanol ether					
2-Thio-5-ethyl	A	24	191–193[f]						
5-Ethyl	B[g]	75	300–303[g]	Methanol					
1,3-Dimethyl-5-ethyl	C	100	81–82	Skellysolve B	$C_8H_{12}N_2O_2$	57.12	57.03	7.19	7.42
5-Propyl	A[d]	1.6	296–298	Methanol	$C_7H_{10}N_2O_2$	54.53	54.03	6.54	6.20
6-Propyl	B[h]	90	221–223	Water	$C_7H_{10}N_2O_2$	54.53	54.38	6.54	6.36
1-Ethyl-6-propyl	[h]	42	162–163	Water, Skellysolve C	$C_9H_{14}N_2O_2$	59.32	59.45	7.74	7.67
1,3-Dimethyl-6-propyl	C	100	60–61 (b.p. 173–176°, 4.5 mm.)		$C_9H_{14}N_2O_2$	59.32	59.65	7.74	7.74
2-Thio-5-butyl	A	13	151–153[i]	Water					
5-Butyl	B	92	291–293	Water, methanol	$C_8H_{12}N_2O_2$	57.12	56.61	7.19	6.94
1,3-Dimethyl-5-butyl	C	94	34–36 (b. p. 116°, 0.06 mm.)		$C_{10}H_{16}N_2O_2$	61.20	61.62	8.22	8.35
2-Thio-5-hexyl	A	15	168–169	Water-methanol, Skellysolve C-acetone	$C_{10}H_{16}N_2OS$	56.57	56.86	7.60	7.53
5-Hexyl	B	90	272–273	Acetone glacial acetic acid	$C_{10}H_{16}N_2O_2$	61.20	61.33	8.22	8.19
5-(β-cyclohexyl)ethyl	A[d]	9	320–322	Isopropanol	$C_{12}H_{18}N_2O_2$	64.84	65.20	8.16	8.20
2-Thio-5-phenyl	A	6	313–315	Isopropanol	$C_{10}H_8N_2OS$	58.80	59.08	3.95	3.87
5-Phenyl	B[j]	97	above 350[j]						
1,3-Dimethyl-5-phenyl	C	61	147–148	Water	$C_{12}H_{12}N_2O_2$	66.65	66.69	5.60	5.50
2-Thio-6-phenyl	[k]	76	259[k]						
6-Phenyl	B	85	269–271[l]	Ethanol					
1,3-Dimethyl-6-phenyl	C	87	121–122[m]	Acetone-Skellysolve B					
2-Thio-5-benzyl	A[n]	9	211–213[n]	Ethanol					
5-Benzyl	B[n]	87	294–296[n]	Glacial acetic acid					
2,4(1H-3H)-Quinazolinedione		...	353–354						
1,3-Dimethyl-2,4(1H, 3H)-quinazolinedione	[k]	80	166–167[o]	Ethanol					
5,5-Diphenylhydrouracil	[k]	76	312–313	Absolute ethanol	$C_{16}H_{14}N_2O_2$	72.16	72.32	5.30	5.26

[a] There appears to be no reference in the literature to the preparation of uracil by the hydrolysis of 2-thiouracil. [b] Prepared from the corresponding commercially available thiouracil. [c] Lit. (5): darkens 280°, m. p. 335°. [d] Prepared by using urea in place of thiourea in Method A. [e] Lit. (8): m. p. 123–124°. [f] Lit. (6): m. p. 191–192°. [g] 5-Ethyluracil has also been prepared by the hydrolysis of 2-ethylmercapto-4-oxo-5-ethylpyrimidine [Johnson, T. B., *J. Biol. Chem.*, **2**, 111(1906). Reported m. p. 300° decompn.] and by the bromination and dehydrobromination of 5-ethylhydrouracil [Tafel, J., and Thompson, H. B., *Ber.*, **40**, 4495(1907). Darkens 300°, m. p. 303°]. [h] See experimental section. [i] Lit. (6): m. p. 151.5–153.5°. [j] Prepared earlier by the hydrolysis of 2-ethylmercapto-4-oxo-5-phenylpyrimidine, reported; does not melt at 350° [Wheeler, H. L., and Bristol, H. S., *Am. Chem. J.*, **33**, 448(1905).] [k] Lit. (7): m. p. 259°. [l] Lit. (7): m. p. 270°. [m] Lit. (15): m. p. 122–122.5°. [n] These compounds were prepared as described by Johnson, T. B., and Ambeland, J. C., *J. Am. Chem. Soc.*, **60**, 2941(1938). Reported: 2-thio-5-benzyluracil, m. p. 210–211°; 5-benzyluracil, m. p. 294–295°. [o] Lit. (11), m. p. 166°.

and 6-methyl-6-phenylhydrouracil (14) and by Johnson and Evans (15) for the preparation of 6-phenylhydrouracil. However, even though several approaches were investigated it was not possible to synthesize the desired isomeric 6,6-diphenylhydrouracil. The latter corresponds to the other possible 6-membered-ring homolog of diphenylhydantoin, i. e., the one in which a methylene group has been inserted between positions 4 and 5.

PHARMACOLOGICAL RESULTS

The compounds listed in Tables I and II were studied by Dr. D. G. Wenzel, of this School, for anticonvulsant potency. The results are published in THIS JOURNAL. Also, Drs. Graham C. Chen and C. R. Ensor, of Parke, Davis and Co., have tested certain of the compounds. They demonstrated significant antimetrazole activity in mice by 1,3-dimethyl-5-ethyluracil, 1,3-dimethyl-6-propyluracil and 1,3-dimethyl-6-phenyluracil. The pharmacological results have encouraged us to undertake further syntheses of uracils.

We wish to acknowledge the helpful cooperation of the pharmacologists.

EXPERIMENTAL

α-Cyano Ureides (Table I).—Ethyl α-cyanobutyrate and ethyl α-cyanocaproate were prepared by alkylation of the sodium salt of ethyl cyanoacetate

TABLE III.—COMPARATIVE PHYSICAL PROPERTIES OF CERTAIN URACILS AND THEIR N-SUBSTITUTED DERIVATIVES

Uracil	m. p., ° C.	Solubility[a]	
		Water	Ether
Uracil	342	Sl. soluble	Sl. soluble
1-Methyl-	237	Soluble	Soluble
1,3-Dimethyl-	121	Very soluble	Insoluble
6-Propyl-	223	Insoluble	Soluble
1-Ethyl-6-propyl-	163	Sl. soluble	Sl. soluble
1,3-Dimethyl-6-propyl	61	Soluble	Sl. soluble
5-Butyl-	293	Insoluble	Soluble
1,3-Dimethyl-5-butyl-	36	Soluble	Sl. soluble
5-Phenyl	>350	Very insoluble	Soluble
1,3-Dimethyl-5-phenyl	148	Sl. soluble	Soluble
6-Phenyl	271	Insoluble	Soluble
1,3-Dimethyl-6-phenyl	122	Sl. soluble	Soluble
2,4(1H,3H)-Quinazolinedione[b]	354	Insoluble	Soluble
1,3-Dimethyl-2,4(1H,3H)-quinazolinedione[b]	167	Sl. soluble	Soluble

[a] The solubility terms are of a descriptive nature only.
[b] May be considered to be a benzouracil.

with ethyl bromide and butyl bromide, respectively, following the procedure of Albertson (4). Small amounts of dialkyl cyanoacetic ester were separated from the monoalkylated product through the insolubility of the former in the aqueous alkali used to saponify the esters. This technique has been used by Hessler to separate diisopropyl and diisoamylcyanoacetic ester from the corresponding monosubstituted products (16). Ethyl α-cyanovalerate was prepared by the reductive alkylation of ethyl cyanoacetate with propionaldehyde, following the procedure of Alexander and Cope (5). The method of Johnson and Bergman (3) was used to prepare the α-cyano ureides of Table I from the appropriate α-cyano acids, which were made by saponification of the α-cyano esters (3, 16).

Attempts to prepare 5-butyluracil through the reductive cyclization of α-cyanocaprolyl urea (3) were thwarted by the insolubility of the ureide in the aqueous media used by these workers; however, by use of 50% ethanol, it was possible to obtain a small amount of the impure substance subsequently identified by a mixed melting point determination with an authentic sample of 5-butyluracil.

General Method A: 2-Thio-5-alkyluracils (Table II).—The procedure described herewith for the preparation of 2-thio-5-ethyluracil is analogous to that used by Anderson and co-workers (6) for the preparation of this compound, except that the reaction period for the formation of the intermediate α-formylbutyric ester was extended to thirty hours and the stirred reaction mixture was kept at 0° in order to avoid a vigorous exothermic reaction. Under these conditions, a 50% excess of freshly purified ethyl formate, in place of the 100% excess used by Anderson and co-workers, was found to be sufficient.

To a suspension of 16 Gm. (0.69 mole, 20% excess) of sodium sand in 150 ml. of dry ether contained in a 1-liter 3-necked flask, equipped with a reflux condenser protected by a drying tube, sealed Hershberg stirrer and dropping funnel there was added over a period of eight hours and with ice bath cooling a solution of 67 Gm. (0.58 mole) of ethyl butyrate and 65 Gm. (0.85 mole, 50% excess) of ethyl formate in 100 ml. of dry ether. The ethyl formate had been freshly purified by distillation over phosphorus pentoxide. The mixture was stirred at 0° for thirty hours, then at 25° for twelve hours, after which the ether and unreacted esters were removed under reduced pressure without the application of heat. A solution of 33 Gm. (0.43 mole) of thiourea in 250 ml. of anhydrous ethanol was then added to the semi-solid residue and the mixture refluxed with stirring for seven hours. The ethanol was then removed by distillation, the last traces being removed under reduced pressure. The solid residue was dissolved in 250 ml. of water and the water washed with 150 ml. of ether. After boiling to expel dissolved ether, the solution was cooled in an ice bath and acidified with concentrated hydrochloric acid to precipitate a yellow resin, which was dissolved in boiling water and treated with charcoal. Cooling caused the separation of 21 Gm. (24% yield) of white 2-thio-5-ethyluracil, m. p. 191–193°. Reported (6): m. p. 191–192° (3.6%). This product was hydrolyzed with 10% aqueous chloroacetic acid without further purification.

The following 2-thio-5-alkyluracils of Table II were obtained by the same procedure: 2-thio-5-butyl, 2-thio-5-hexyl, 2-thio-5-phenyl, and 2-thio-5-benzyluracil. In the case of the 2-thio-5-hexyl derivative, the only instance in which it was tried, it was found that the reaction time for the initial formulation could be cut to twenty-four hours, instead of approximately forty-two hours, if a few ml. of absolute ethanol were added to the reaction mixture at the start.

5-Propyluracil and 5-(β-cyclohexyl)ethyluracil were prepared by the foregoing method, except that urea was used in place of thiourea in the condensation with the α-formyl esters.

General Method B: Uracils from 2-Thiouracils.—Uracil was conveniently obtained by the hydrolysis of commercially available 2-thiouracil following the procedure which Johnson and Hemingway used for the hydrolysis of 2-thio-6-phenyluracil (7). A suspension of 95 Gm. (0.74 mole) of 2-thiouracil in 1400 ml. of 10% aqueous chloroacetic acid was

heated under reflux for six hours, during which time the 2-thiouracil dissolved. The solid obtained upon cooling was recrystallized from water; it yielded 80 Gm. (97% yield) of a white crystalline solid, m. p. 339–351°. Lit. (17): darkens 280°, m. p., 335°.

This procedure was also followed for the hydrolysis of the following alkyl- or phenyl-2-thiouracils of Table II: 2-thio-5-ethyl-, 2-thio-6-propyl, 2-thio-5-butyl-, 2-thio-5-hexyl-, 2-thio-5-phenyl- and 2-thio-5-benzyluracil.

2-Thio-6-phenyluracil (Table II.)—The procedure of Johnson and Hemingway (7) was used for the preparation of 2-thio-6-phenyluracil, except for the additional modification of mechanical stirring during the condensation of thiourea with ethyl benzoylacetate. The crude reaction product, m. p. 259°, was hydrolyzed to 6-phenyluracil without further purification.

6-Phenyluracil (Table II).—Johnson and Hemingway accomplished the hydrolysis of 2-thio-6-phenyluracil using aqueous chloroacetic acid (7). However, due to the insolubility of the thiouracil in this medium, it was found to be more expedient to use 40% hydrobromic acid.

A mixture of 32 Gm. (0.16 mole) of 2-thio-6-phenyluracil and one liter of 40% hydrobromic acid was refluxed for twenty hours, during which time all the 2-thio-6-phenyluracil dissolved. The solution was then cooled, poured into 1.5 Kg. of cracked ice, whereupon 25 Gm. (85% yield) of a white solid was removed by filtration. Recrystallization from ethanol yielded a pure product, m. p. 269–271°. Lit. (7): m. p. 270°.

General Method C: 1,3-Dimethyluracils.—1,3-Dimethyl-6-phenyluracil is described by Evans and Johnson (15). However, no experimental details are given, except that it is prepared by the dimethyl sulfate methylation of 6-phenyluracil.

To a solution of 15 Gm. (0.081 mole) of 6-phenyluracil dissolved in 3 L. of 2% aqueous sodium hydroxide was added 40 Gm. (0.32 mole) of dimethyl sulfate over a period of one-half hour with vigorous mechanical stirring. The mixture was stirred at room temperature for an additional two hours, ammonium hydroxide was added and the solution cooled in an ice bath causing the separation of a white solid which was removed by filtration. It was recrystallized from acetone-petroleum ether to yield 15 Gm. (87% yield) of 1,3-dimethyl-6-phenyluracil, m. p. 121–122°. Lit. (15): m. p. 122–122.5°.

Other 5- or 6-alkyl or aryluracils of Table II were methylated in the same manner. In some cases, the methylated product did not separate from the reaction mixture upon cooling because of its water solubility. In such cases, the reaction mixture was extracted with chloroform, the chloroform removed and the residue recrystallized from petroleum ether.

1-Ethyl-6-propyluracil (Table II).—A 300% excess of diethyl sulfate was added to 5 Gm. of 6-propyluracil dissolved in 100 ml. of 10% aqueous sodium hydroxide. The mixture was stirred at room temperature for three days, ammonium hydroxide was added and the solution stirred for an additional two days. The alkylated product was isolated by ether extraction and recrystallized successively from water and Skellysolve® C to yield 2.5 Gm. (42% yield) of a white solid, m. p. 162–163°.

That only one ethyl group was introduced is shown by the fact that the product is only slightly soluble in cold water, but it dissolves readily in 5% aqueous sodium hydroxide. Asssignment of the ethyl group to the 1 position is based upon the work of Hilbert and Johnson (9) who determined that the N_1 position was alkylated preferentially over the N_3 position in the case of 2,4-dimethoxypyrimidine.

Ethyl Diphenylcyanoacetate.—This compound has been described by Bockmühl (12). However, since he gave only very brief experimental details, its preparation is recorded in detail, as follows.

To 19 Gm. (0.083 mole) of sodium, sand and 140 ml. of dry benzene contained in a 1-liter 3-necked flask equipped with nitrogen inlet, sealed Hershberg stirrer, dropping funnel, thermometer and reflux condenser and protected by a drying tube, there was slowly added with constant mechanical stirring over a period of five hours a solution containing 58 Gm. (0.3 mole) of diphenylacetonitrile, 42 Gm. (0.38 mole) of chlorobenzene and 50 ml. of benzene sufficiently warm to insure solution. Warming with infrared heat was sufficient to initiate an exothermic reaction which was maintained at 36–40° by controlling the rate of addition and by the use of a water bath maintained at 30°. The stirring was continued during the entire course of the preparation. At the end of the five-hour period, the mixture was warmed to 50° for an hour, cooled in an ice-salt mixture and 42 Gm. (0.39 mole) of freshly distilled ethyl chlorocarbonate was added over a period of an hour and a half, so that the temperature did not rise above 10°. During this time it was necessary to add an additional 150 ml. of benzene in order to keep the mixture in a semi-fluid state. The reaction mixture was then heated at reflux for an hour, cooled, and 20 ml. of absolute methanol was added to remove any unreacted sodium present. The entire reaction mixture was then diluted with 500 ml. of benzene and extracted with 500 ml. of ice cold water, the water back-washed with benzene and the combined benzene solutions dried over sodium sulfate for sixteen hours. (The slightly alkaline aqueous solution was acidified and extracted with ether, the ether dried and removed to yield about 1 Gm. of a tarry material which was discarded.) The benzene solution was concentrated *in vacuo* to yield a syrup which solidified upon cooling, m. p. about 60°. Recrystallization of the latter from methanol (1 Gm. insoluble, m. p. 180–185°), accompanied by treatment with charcoal, yielded 51 Gm. (65% yield) of ethyl diphenylcyanoacetate, m. p. 59–61°. Lit. (12): m. p. 58–59°, 57% yield.

Ethyl α,α-Diphenyl-β-aminopropionate Hydrochloride.—Bockmühl accomplished the catalytic reduction of ethyl diphenylcyanoacetate to ethyl α,α-diphenyl-β-aminopropionate over nickel catalyst (12). We have found that the amino ester could be conveniently isolated, purified and used in the next reaction in the form of the hydrochloride.

A suspension of fresh Raney nickel catalyst, prepared from 10 Gm. of nickel-aluminum alloy, in a solution containing 43 Gm. (0.16 mole) of ethyl diphenylcyanoacetate, with sufficient methanol to make a total volume of 140 ml., was shaken with hydrogen at a mean pressure of 1200 p.s.i. (25°) at a

temperature of 105–140°. The calculated amount of hydrogen was absorbed, after which the reaction mixture was cooled, the catalyst removed by filtration and the resulting solution concentrated under reduced pressure. Absolute ethanol was twice added and removed to eliminate the last traces of methanol. The syrupy residue was diluted with petroleum ether and hydrogen chloride gas added to precipitate an oil which solidified. There was obtained 32 Gm. (66% yield) of a white solid, m.p. 184–186°, after trituration with acetone. It was recrystallized for analysis from a mixture of ethyl acetate and ethanol, m.p. 191.5–192.5°.

Anal.—Calcd. for $C_{17}H_{19}NO_2 \cdot HCl$: Cl, 11.59. Found: Cl, 11.70.

5,5-Diphenylhydrouracil (Table II).—A solution of 5 Gm. (0.016 mole) of ethyl α,α-diphenyl-β-aminopropionate hydrochloride, dissolved in 50 ml. of water, was added to a solution of 2 Gm. (0.025 mole) of potassium cyanate in 25 ml. of water. The solutions were mixed well and heated on the steam bath for one hour, after which 50 ml. of concentrated hydrochloric acid was added and the resulting solution refluxed for twenty hours. Cooling caused the separation of 3.3 Gm. (76% yield) of 5,5-diphenylhydrouracil, m. p. 312–313°.

2,4 (1,3)-quinazolinedione (Table II) was prepared by the procedure of Bogert and Scatchard utilizing the reaction of potassium cyanate with anthranilic acid (10).

1,3-Dimethyl-2,4 (1,3)-quinazolinedione (Table II).—To a solution of 15 Gm. (0.093 mole) of the crude sodium salt of quinazolinedione (*vide supra*) dissolved in 500 ml. of 5% aqueous sodium hydroxide at 70°, there was added dropwise with vigorous mechanical stirring, 70 ml. of dimethyl sulfate. A white solid precipitated while the solution was still alkaline. The resulting mixture was back titrated with sufficient 15% sodium hydroxide solution to render it distinctly alkaline, after which it was cooled in an ice bath and the solid which had separated was collected on a funnel, m. p. 166–167°.

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Anticonvulsant Activity of Some Uracils and Related Compounds*

By DUANE G. WENZEL

Seventeen uracils and five related compounds were tested for their ability to protect mice from electroshock and Metrazol® convulsions. It was possible to demonstrate a limited structure-activity relationship for the electroshock testing. Such a relationship was not apparent from the chemoshock results.

IT WAS RECENTLY REPORTED that uracil and some derivatives possess anticonvulsant properties against electroshock seizures (1). On the basis of this information a number of substituted uracils and related structures were synthesized.[1] The synthesis of the new compounds has been reported in THIS JOURNAL. This paper concerns the testing of these compounds as anticonvulsants. The quinazolinediones tested are benzouracils and the aliphatic acid derivatives of urea are intermediates in the synthesis of certain of the uracils. It was thought that the ureides might resemble biotransformation products of the uracils.

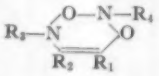
* Received May 5, 1955, from the School of Pharmacy, University of Kansas, Lawrence.

[1] The author thanks Dr. J. H. Burckhalter and H. C. Scarborough, Jr., for the compounds tested.

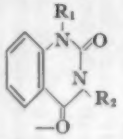
EXPERIMENTAL

Materials.—Three of the compounds tested, 1,3-dimethyluracil, 1,3-dimethyl-5-ethyluracil, and 1,3-dimethyl-6-propyluracil, were water-soluble. The other substances were relatively insoluble and prepared as aqueous suspensions with 0.5% methylcellulose, 4000 c.p.s.

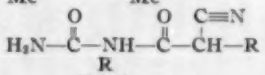
TABLE I.—COMPARATIVE ANTICONVULSION PROTECTION OF URACILS AND RELATED STRUCTURES[a]



	R_1	R_2	R_3	R_4	Maximal Electroshock Protection, Time in Minutes: 30	90	150	210	270	Metrazol® protection[b]
1.	Ethyl	H	H	H	2.35 (1.50–3.52)	2.21 (1.33–3.65)	4.87 (2.91–7.92)	—[c]	—[c]	—[c]
2.	Propyl	H	H	H	1.73 (1.17–2.46)	2.14 (1.54–2.86)	3.51 (2.41–5.07)	4.66 (3.00–7.08)	—[c]	1.55 (1.13–2.12)
3.	Butyl	H	H	H	1.16 (0.71–1.42)	1.75 (1.09–2.63)	3.72 (2.24–6.10)	4.01 (2.12–8.73)	—[c]	—[c]
4.	Hexyl[d]	H	H	H	...	...	...	...	...	2.20 (1.21–4.00)
5.	Phenyl	H	H	H	5.46 (3.70–7.78)	4.24 (3.75–4.70)	5.39 (2.65–10.3)	—[c]	—[c]	—[c]
6.	Benzyl	H	H	H	1.95 (1.65–2.18)	2.03 (1.61–2.48)	2.98 (2.23–3.77)	4.41 (3.14–6.16)	6.07 (2.80–12.9)	—[c]
7.	β-(Cyclohexyl) ethyl	H	H	H	3.23 (2.52–4.06)	4.87 (3.61–6.38)	6.04 (4.84–7.44)	—[c]	—[c]	3.38 (2.33–4.90)
8.	H	Pr	H	H	2.48 (1.43–3.96)	2.32 (1.55–3.40)	2.71 (1.94–3.75)	3.30 (1.80–5.01)	3.54 (2.57–4.76)	—[c]
9.	H	Ph	H	H	1.54 (0.99–2.26)	1.19 (0.55–2.21)	1.56 (0.99–2.26)	2.10 (1.23–3.57)	2.44 (1.65–3.48)	3.13 (1.77–5.42)
10.	H	H	Me	H	3.92 (2.69–5.65)	—[c]	—[c]	—[c]	—[c]	—[c]
11.	H	H	Me	Me	2.86 (1.84–4.26)	3.90 (2.80–5.46)	3.41 (2.43–4.76)	4.94 (3.86–6.22)	—[c]	—[c]
12.	Ethyl	H	Me	Me	1.15 (0.74–1.78)	1.53 (1.15–1.95)	1.48 (1.16–1.68)	2.11 (1.59–2.79)	2.76 (1.80–4.05)	1.87 (1.16–2.79)
13.	Butyl[e]	H	Me	Me						
14.	Phenyl[d]	H	Me	Me	1.86 (1.36–2.37)	1.13 (0.86–1.48)	1.23 (1.01–1.48)	1.23 (1.01–1.48)	1.85 (1.44–2.36)	...
15.	H[e]	Pr	Me	Me	...	...	...	...	...	5.16 (2.71–9.80)
16.	H[e]	Ph	Me	Me	0.65 (0.44–0.96)	0.45 (0.20–0.99)	0.45 (0.20–0.99)	2.44 (1.50–3.84)	2.13 (1.23–3.57)	—[c]
17.	Diphenyl	2H	H	H	—[c]	—[c]	—[c]	—[c]	—[c]	1.49 (1.17–1.89)



	R_1	R_2								
18.	H	H			—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
19.	Me	Me			2.00 (1.60–2.50)	1.65 (1.51–1.80)	1.67 (1.49–1.90)	1.68 (1.50–1.88)	1.89 (1.78–2.00)	—[c]



	R									
20.	Ethyl				4.59 (2.69–7.52)	1.52 (1.04–2.25)	—[c]	—[c]	—[c]	—[c]
21.	Propyl				1.28 (0.85–1.92)	0.95 (0.71–1.27)	1.20 (0.96–1.61)	1.48 (1.15–1.91)	—[c]	—[c]
22.	Butyl				1.42 (1.77–2.66)	1.56 (0.81–2.77)	2.21 (1.52–3.23)	2.67 (1.66–4.05)	6.22 (2.07–18.6)	—[c]

[a] PD_{50} expressed as mM/Kg. Values in parentheses represent 95 per cent confidence limits. [b] One hour after administration of test compound. [c] Inactive or less than 50 per cent protection. [d] Moderate to severe depression at effective levels. [e] Insufficient compound to complete tests.

Methods.—Male albino mice weighing from 15 to 25 Gm. were used as the experimental animals. All compounds were administered orally with a stomach needle. Animals were fasted for six to eight hours prior to the administration of the test substances. Eight mice were used for each dose and 4 to 6 doses of each compound were tested. Dose levels were arranged in a geometric progression. For most of the compounds a factor of 1.5 resulted in a satisfactory range. The maximum dose tested for any compound was 7.6 mM/Kg. If the proper range could not be obtained including this dose or less, the compound was considered to be inactive.

Maximal Electroshock Seizures.—Convulsive seizures were produced by delivering a half-wave 60-cycle alternating current of 24 ma. for 0.2 second through ear electrodes. In order to determine the duration of activity as well as the peak anticonvulsant effect, the animals were shocked at thirty, ninety, one hundred and fifty, two hundred and ten, and two hundred and seventy minutes following the administration of the drug. Protection against the tonic extensor phase of the convulsion was the criterion of the test. The PD_{50} as millimoles per kilogram was determined for each compound at each of the times tested. Results were obtained by the graphic estimation method of Litchfield and Wilcoxon (2).

Metrazol® Seizures.—Administration of the test compounds was followed in one hour by Metrazol®, 85 mg./Kg. subcutaneously. This dose has been determined as the CD_{97} for mice (3). Positive protection against chemoshock was prevention of tonic extension of the hindlegs and/or clonic spasm lasting for more than five seconds. The results were derived in the same manner as those of maximal electroshock seizures.

RESULTS AND DISCUSSION

From the results as shown in Table I (Nos. indicated) it may be seen that certain compounds have no values listed for the electroshock test. Both 1,3-dimethyl-5-butyluracil, 13, and 1,3-dimethyl-6-propyluracil, 15, exhibited marked toxicities at the effective dose range and thus could not be evaluated. The usual toxic manifestation noted with these two compounds and at certain higher dose levels of others was moderate to severe depression. This was particularly apparent with the higher doses of the cyanoureides, 20–22, 1,3-dimethyl-5-ethyluracil, 12, and 1,3-dimethyl-6-phenyluracil, 16. An unusual effect of depression combined with a hypersensitivity to touch and a stiff-legged posture was noted with the higher doses of the 1,3-dimethyl compounds.

Electroshock Results.—As seen in Table I, the usual pattern of protection was peak activity in the period of thirty minutes to two and one-half hours. It also may be observed that electroshock activity increased with the size of the aliphatic substituent in the 5-position (R_1). Although 5-hexyl uracil, 4, was incompletely tested, it appeared to fall in line with this trend.

It is interesting to compare the differences in structure-activity relationships of the uracils and the barbiturates. The effective barbiturates all are disubstituted in the 5-position but the uracils have only one replaceable hydrogen at this position. While the 5-phenyl group is almost of critical importance to electroshock activity in the barbiturates this is obviously not true for the uracils. The only compound tested with disubstitution in the 5-position, 5,5-diphenyluracil, 17, was ineffective for electroshock protection. A comparison of 5-phenyluracil, 5, with the other 5-substituted compounds shows it to be relatively inactive. It is particularly significant that the 5-benzyl compound, 6, should be considerably more active than the 5-phenyl.

From the limited information that may be derived from the two 6-substituted (R_2) uracils, 8 and 9, it would appear that this change (6-substitution) may be an improvement over 5-substitution. Particularly evident is the difference between the phenyl derivatives.

The 1-methyluracil, 10, showed little electroshock action but this was improved by a second methyl group on the 3-position, 11. Further substitution of the dimethyluracil with a 5-ethyl substituent, 12, increased the activity. It was noted that 1,3-substitution generally increased the toxicity over that of the corresponding 5- or 6-substituted uracils. This was particularly evident with 1,3-dimethyl-5-butyluracil, 13, and 1,3-dimethyl-6-propyluracil, 15. The methyl substitution somewhat improved the activity of the 5- and 6-phenyluracils.

Unsubstituted 2,4-quinazolinedione, 18, showed no trace of activity, but the 1,3-dimethyl derivative provided substantial protection without any observed toxicity.

The activity of the α-cyanoureides, 20–22, increased with the increase in molecular weight. All these compounds were quite toxic, however. Only a small margin was observed between the protective and toxic doses.

Metrazol® Protection.—In the 5-substituted series the propyl, 2, and hexyl, 4, derivatives were particularly effective. The 5-ethyl, 1, and 5-butyl, 3, compounds showed some activity at the highest doses tested (7.6 mM/Kg.), but not enough to obtain protective values. While the 5-propyluracil, 2, was very active, the corresponding 6-derivative, 8, was almost without effect.

Dimethyl substitution was generally observed to increase the activity, but the accompanying increase in toxicity as in the case of 1,3-dimethyl-6-phenyluracil, 16, resulted in relatively low activity.

The quinazolinediones and α-cyanoureides were without effect in the chemoshock tests, although 1,3-dimethyl-2,4-quinazolinedione, 19, appeared to delay the onset and modify the severity of Metrazol® seizures.

SUMMARY

1. Seventeen uracils, two benzouracils and three α-cyanoureides were tested for their ability to prevent electroshock and Metrazol® seizures in mice.

2. Most of the compounds were active in the electroshock test and there were indications that alkyl substituents increased the activity with

the size of the substituent group. The introduction of 1,3-dimethyl groups also appeared to improve activity.

3. Although several of the uracils were effective in preventing chemoshock, there was no obvious relationship of structure to activity.

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The Alkaloids of *Rauwolfia serpentina* Benth*

By DONALD D. PHILLIPS† and MOHINDRA S. CHADHA‡

Rauwolfia **is an important genus of the plant family *Apocynaceae*. There are nearly one hundred and twenty-five species of *Rauwolfia* which are distributed all over the tropical regions of the world (1–8). The most important member of the genus is *R. serpentina* Benth; the crude drug was known to the ancient Indians as a useful febrifuge (8), a remedy for snake bite, and as a cure for dysentery. In more recent times it has been used for insomnia, hypochondria and insanity.**

The presence of alkaloidal principles in *R. serpentina* was first pointed out in 1890 by Greshoff (9). In 1933, Chopra, Gupta, and Mukherjee (10) reported the hypotensive activity of the material extracted from the plant and in 1931 Siddiqui and Siddiqui (11) isolated a series of crystalline alkaloids from *R. serpentina*. Active chemical and pharmacological interest in *R. serpentina* has resulted in the discovery of several new alkaloids (Table I). The isolation by Müller, Schlittler, and Bein (12) of reserpine, an alkaloid with pronounced hypotensive and sedative activity, has lent further impetus to the pharmacological and chemical study of the alkaloids of *R. serpentina*. A brief summary of the chemical aspects of *R. serpentina* will be presented in this review.[1]

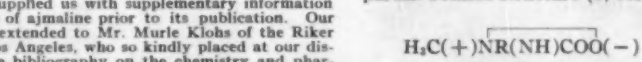
THE CHEMISTRY OF THE ALKALOIDS FROM R. SERPENTINA

THE alkaloids that have been isolated from *R. serpentina* to date are listed together with their pertinent physical properties in Table I. With the exception of thebaine and papaverine recently isolated by Hofmann (20), all of the known alkaloids from *R. serpentina* are indole bases. Included in the group are strong, moderately strong, and weak bases; the strongly basic alkaloids are deep yellow in color while the others are colorless. Schlittler, *et al.* (28), have found it convenient to subdivide the alkaloids of *R. serpentina* according to their chemical structures. In a similar manner, the alkaloids of *R. serpentina* will be considered in this review under the headings:

I. Tertiary indoline alkaloids
II. Quaternary anhydronium bases
III. Tertiary indole bases of the yohimbine type
IV. Tertiary indole bases of the tetrahydroalstonine type
V. Alkaloids of unknown ring structure
VI. Non-indole alkaloids

I. **Tertiary Indoline Alkaloids.**—The alkaloids belonging to this group are ajmaline, iso- and neoajmaline, and rauwolfinine. The available evidence indicates that ajmalinine belongs to this group but because of the limited chemical data it will be considered under Group V.

(*a*) *Ajmaline.*—Ajmaline, $C_{20}H_{26}O_2N_2$, was first isolated by Siddiqui and Siddiqui (11) and almost simultaneously by van Itallie and Steenhauer (13). The latter workers ascribed to it the molecular formula $C_{21}H_{28}O_2N_2$. The recent observations of Robinson and his coworkers (29) however, have confirmed the molecular formula, $C_{20}H_{26}O_2N_2$, originally proposed by Siddiqui and Siddiqui. The early Indian workers (11) reported: (*a*) the absence of hydroxyl, methoxyl, and methylenedioxy groups, (*b*) the formation of a monobenzoate and hence the presence of a secondary nitrogen, (*c*) the presence of an *N*-methyl group. They presumed that the methyl and the imino groups were both linked to the same nitrogen atom and proposed the partial betaine structure (I) for ajmaline.



I

In 1949, Robinson and collaborators (29) showed that ajmaline was a monoacidic, ditertiary base with strychnidine-like properties. One of the nitrogens was in the form of an *N*-methyl attached

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It is a pleasure to acknowledge the assistance of Sir Robert Robinson who supplied us with supplementary information on the structure of ajmaline prior to its publication. Our thanks are also extended to Mr. Murle Klohs of the Riker Laboratories, Los Angeles, who so kindly placed at our disposal a complete bibliography on the chemistry and pharmacology of *Rauwolfia serpentina*. The splendid cooperation of Drs. E. Schlittler, A. Stoll, A. Hofmann, and N. Neuss is also gratefully acknowledged.
[1] The chemistry and pharmacology of *Rauwolfia* alkaloids has been the subject of several recent reviews (15A, 28, 76, 177, 178, 92). Considerable progress in the field has resulted since the appearance of these articles making a more comprehensive review very desirable.

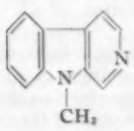
TABLE I.—ALKALOIDS OF *R. Serpentina Benth*

Name	Molecular formula	Sp. Rotation	m. p.	Derivatives	First Reported by
Ajmaline	$C_{20}H_{26}O_2N_2$	$[\alpha]_D^{33} = +128°$ ($CHCl_3$)	158–160°	B. HCl 253–255°; B. Picrate 126–127°	
Ajmalinine	$C_{20}H_{26}O_2N_2$	$[\alpha]_D^{33} = -97°$ ($CHCl_3$)	180–181°	B.HCl 240–245° (decompn); B. Picrate 200–205°	
Ajmalicine	$C_{21}H_{24}O_2N_2$	$[\alpha]_D^{33} = -48.5°$ (C_5H_5N)	250–252°	B. HCl 260–3° (decompn.); B. Picrate 212–215° (decompn.)	S. Siddiqui and R. H. Siddiqui (11)
Serpentine	$C_{21}H_{22}O_3N_2$	$[\alpha]_D^{40} = +188°$ (H_2O)	157–158°	B.HCl 260–261° (decompn.); B. Picrate 261–262° (decompn.)	
Serpentinine	$C_{21}H_{22}O_3N_2$ or $C_{21}H_{26}O_3N_2$		263–265°	B. HCl 260–262°; B. Picrate 225–227°	
"New Alkaloid"			220°		
"Amphoteric Alkaloid"			234°		
Isoajmaline	$C_{20}H_{26}O_2N_2$	$[\alpha]_D^{35} = +72.8°$ (C_2H_5OH)	264–265°	B. H_2PtCl_6 227–228° (decompn.)	S. Siddiqui (11A)
Neoajmaline	$C_{20}H_{26}O_2N_2$		205–207°		
Alkaloid C = ajmalinine		$[\alpha]_D = -76.4°$	177°	.....	
Rauwolfine = ajmaline		$[\alpha]_D = +131.1°$	160°	.....	L. vanItallie and A. J. Steenhauer (13)
Isorauwolfine = isoajmaline		$[\alpha]_D = +75°$	263–265°	.....	
Rauwolfinine	$C_{19}H_{26}O_2N_2$	$[\alpha]_D^{32} = -34.7°$	235–236°	B. HCl, 195° (decompn.)	A. Chatterjee and S. Bose (14)
Reserpine	$C_{33}H_{40}O_9N_2$	$[\alpha]_D^{33} = -117$ to $-118°$ ($CHCl_3$)	262–266° (corr. 277–278°)	B.HCl, H_2O 224° (decompn.); B. Picrate, H_2O 183–186° (decompn.)	J. M. Müller, E. Schlittler, and H. J. Bein (12)
Deserpidine (canescine)	$C_{22}H_{28}O_5N_2$	$[\alpha]_D^{35} = -137° \pm 1$ ($CHCl_3$)	228–232°	.....	E. Schlittler, P. R. Ulsafer, M. L. Pandow (77B)
Rescinnamine[a]	$C_{35}H_{42}O_9N_2$	$[\alpha]_D^{24} = -97° \pm 2$ ($CHCl_3$)	238–239°	.....	M. W. Klohs, M. D. Draper, and F. Keller (24)
Reserpinine		$[\alpha]_D^{20} = -98°$ ($CHCl_3$)	224–226°	.....	E. Haack, A. Popelak, H. Spingler, and F. Kaiser (23)
Sarpagine[a]	$C_{19}H_{22}O_2N_2$	$[\alpha]_D^{30} = +54°$ (C_5H_5N)	320°	B.HCl, 220° (decompn.)	A. Stoll and A. Hofmann (17)
Raupine	$C_{20}H_{24}O_2N_2$ ($C_{19}H_{22}O_2N_2 + CH_3OH$)	$[\alpha]_D^{30} = +63°$ (CH_3COOH)	325°	B. HCl, 239°	K. Bodendorf and H. Eder (18)
Serpenine			315°		S. Bose (16)
Substance I		$[\alpha]_D^{30} = -123°$ ($CHCl_3$)	228°	B.HCl, 258–263° (decompn.)	A. Popelak, H. Spingler, and F. Kaiser (19)
= Raubasinine			228°		E. Haack, A. Popelak, H. Spingler, and F. Kaiser (23)
= Alkaloid C	$C_{21}H_{24}O_4N_2$	$[\alpha]_D^{20} = -127°$ (C_5H_5N)	240°	B.HCl, 263–264° (decompn.)	A. Hofmann (20)
= New Alkaloid		$[\alpha]_D^{33} = -125°$ ($CHCl_3$)	240–241°		F. L. Weisenborn, M. Moore, and P. A. Diassi (21)
= Reserpinine[a]		$[\alpha]_D^{33} = -117° \pm 4$ ($CHCl_3$)	238–239°	B. HCl, 244–246°	E. Schlittler, H. Saner and J. M. Müller (22)
= Alkaloid A					N. Neuss, H. E. Boaz, and J. W. Forbes (26c)

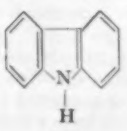
Name	Molecular formula	Sp. Rotation	m. p.	Derivatives	First Reported by
Substance II		$[\alpha]_D^{25} = -61°$ (CHCl₃)	247–248° (corr. 255°)		(19)
= Raubasine					(23)
= δ-yohimbine	$C_{21}H_{26}O_3N_2$	$[\alpha]_D^{25} = -45°$ (C_5H_5N)	257°	B. HCl, 280–290° (decompn.)	(20)
= Alkaloid P		$[\alpha]_D^{20} = -37° \pm 6$ (CH_3OH)	253–254°	B. HCl, 264–265°	(26c)
= Ajmalicine[a] (*py*-tetrahydroserpentine)		$[\alpha]_D^{24} = -58.1° \pm 2$ ($CHCl_3$)	250°	B. HCl, 265–268°	(11), M. W. Klohs, M. D. Draper, F. Keller, W. Malesh, F. J. Petracek (62)
Reserpiline	$C_{22}H_{28}O_5N_2$	$[\alpha]_D^{24} = -40° \pm 2$ (C_5H_5OH)	amorphous	B. HCl, 205–207°	M. W. Klohs, *et al.* (24A)
Rauhimbine (corynanthine)	$C_{21}H_{28}O_3N_2$	$[\alpha]_D^{20} = -82°$ (C_5H_5N)	218–225°	B. HCl, $2H_2O$ 285°	A. Hofmann (20, 25)
Isorauhimbine	$C_{21}H_{28}O_3N_2$	$[\alpha]_D^{25} = -104°$ (C_5H_5N)	225–228°	B. HCl, 235–250° (decompn.)	A. Hofmann (25)
Yohimbine	$C_{21}H_{26}O_3N_2$	$[\alpha]_D^{25} = +105°$ (C_5H_5N)	235–237°	B. HCl, 300–302°	
Methyl reserpate	$C_{23}H_{30}O_6N_2$	$[\alpha]_D^{25} = -106°$ (C_5H_5N)	244–245°	B. HCl, 219–228°	A. Hofmann (20)
Thebaine	$C_{19}H_{21}O_3N$	$[\alpha]_D^{25} = -279°$ (C_5H_5N)	195°	B. Picrate, 217°	
Papaverine	$C_{20}H_{21}O_4N$		147°	B. HCl, 225–226°	
Serpine	$C_{21}H_{26}O_3N_2$	$[\alpha]_D^{25} = +70.1°$ (C_5H_5N)	213°	B. HCl, 263–264° (decompn.)	A. Chatterjee and S. Bose (27)
Alkaloid 3078[b]	$C_{21}H_{26}O_3N_2$	$[\alpha]_D^{26} = -90°$ (C_5H_5N)	125–128° and 181–183°	B. HCl, 235–240° (decompn.)	F. E. Bader, D. F. Dickel, R. A. Lucas, E. Schlittler (22A)
Chandrine	$C_{20}H_{22}O_3N_2$		230–231°	B. Picrate, 180°	B. Rakshit (179)

[a] Name suggested for future use (180). [b] Since shown to be identical with 3-epi-α-yohimbine (272).

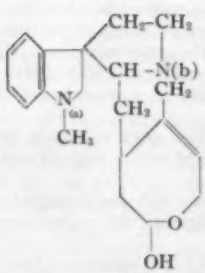
to an aromatic nucleus with a free and reactive *para* position. Ajmaline gave seemingly positive chemical but negative spectral evidence for the presence of an aldehyde group, and a latent aldehyde group was assumed to be present. Evidence for a hydroxyl group and an isolated double bond was also presented. When distilled with soda lime or zinc dust, ajmaline produced Ind-N-methylharman (II) and carbazole (III).



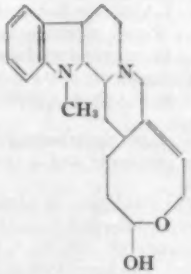
II



III



IV

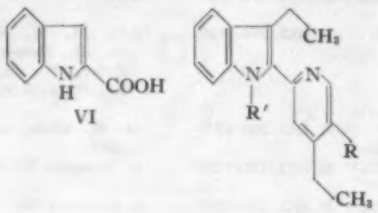


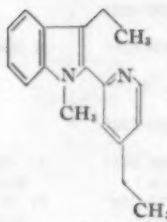
V

Reasoning from the above facts Robinson and collaborators suggested (IV) and (V) as possible structures for ajmaline. They favored structure (IV) as a working hypothesis for the reason that dihydroindoles such as (V) are very uncommon in nature and also because (IV) is related to strychnine and could arise biogenetically according to Woodward's scheme (30). The formation of Ind-N-methylharman (II) was rationalized as a migration of the type $-CRR_1-\overset{|}{C}-H \rightarrow -CR=CR_1-$, and carbazole (III) was said to be the result of a dehydrogenation of an open hexane chain to an aromatic system. Ind-N-methylharman (II) could arise from formula (V) without migration but the formation of carbazole (III) could not be readily explained.

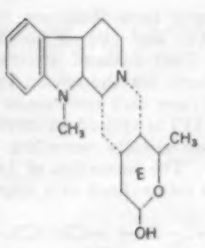
Chatterjee and Bose (31, 32) have studied the infrared spectrum of ajmaline and have reported an absorption band at 5.82μ that indicated 15 to 20% carbonyl absorption, thus confirming the presence of the cyclic-acetal group suggested by Robinson and co-workers (29). The spectrum also contained the band at 7.24μ characteristic of a C-methyl group as well as the typical ether absorption at 9μ. On alkaline fusion of ajmaline, they obtained a crystalline base and two acids, one of which was shown to be indole-2-carboxylic acid (VI), a result that could not be rationalized on the basis of (IV) for ajmaline. Moreover, it was suggested (31, 32) that if structure (V) correctly represented

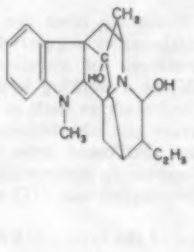
ajmaline, selenium dehydrogenation should produce Ind-N-methylalstyrine (VII, R′ = CH_3, R = C_2H_5), desethyl-Ind-N-methylalstyrine (VIII) or desmethyl-Ind-N-methylalstyrine (VII, R = R′ = CH_3). Chatterjee and Bose could not isolate any of these compounds from the dehydrogenation products of ajmaline but found only Ind-N-methyl-

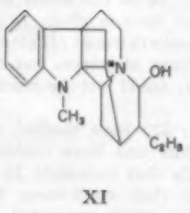


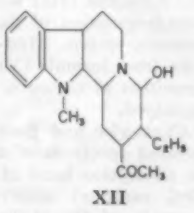


harman (II). For this reason, they suggested structure (IX) for ajmaline, ring D being involved in some undefined weak linkage, so that Ind-N-methylharman was easily formed.









Structure (IX) represents a dihydroindole or dihydroindolenine derivative and similar compounds have been found in nature, e.g., the alkaloids of erythrina (33, 34). Furthermore, a possible biogenesis for such derivatives has been suggested by Schöpf, *et al.* (35). Ring E was presumed to be a six-membered heterocycle similar to that found in alstonine (36, 37) and serpentine (38). Chatterjee and Bose considered the possibility that the C-methyl was in ring B [*cf.* physostigmine (39) or calycanthidine (40)] but preferred to locate it in ring E as it is found in alstonine.

On the basis of more recent studies Robinson and co-workers (41, 41A) have modified their earlier structures (IV and V) and have advanced structure (X) as a better representation for ajmaline. The positive response in the Angeli-Rimini reaction has now been attributed to the intervention of acetaldehyde as a possible link between ajmaline and benzenesulfonylhydroxylamine. The reducing action of ajmaline has been ascribed to a :NCH(OH) group which was recognized by (*a*) the transformation of ajmaline oxime into a nitrile, (*b*) the conversion of N-methylajmaline (which showed a carbonyl band in the infrared spectrum) into monodesoxyajmaline, $C_{20}H_{26}ON_2$ (:N—CH_3—), (*c*) by the production of desoxydihydroajmaline (>NH CH_3—) and desoxyoctahydroajmaline in the Wolff-Kishner reduction of ajmaline and hexahydroajmaline respectively. The strong basic character of ajmaline and the acylable hydroxyl were attributed to the >NCH(OH) group, although these properties have not hitherto been associated with carbinol amines.

The formation of methyl ethyl ketone on chromic acid oxidation of desoxydihydroajmaline was taken as evidence for the presence of —$CH(CH_3)C_2H_5$ in the reduced base. The appreciable increase in the C-methyl value that occurred when ajmaline was converted to desoxydihydroajmaline further suggested the presence of the —NCH(OH)CHEt moiety in ajmaline.

The second active hydrogen was also shown to be part of a hydroxyl group by formation of diacetylajmaline and from a study of the infrared spectrum of desoxydihydroajmaline. The stability of desoxyoctahydroajmaline towards chromic acid suggested that the second hydroxyl was tertiary, and because of its resistance to dehydrating agents, it was placed at the apex of a bridgehead structure. Robinson and co-workers (41, 41A) could find no spectral evidence for the carbonyl group (5.82μ), the C-methyl group (7.24μ) or the ether bridge (9.0μ) that had been reported by Chatterjee and Bose (31, 32). Furthermore, Robinson and colleagues (41, 41A) have also offered a possible biogenetic scheme for the formation of (X) from (XII).

In a private communication (42) Robinson has further elaborated on the chemistry of ajmaline and has suggested a new structure for this alkaloid. The new facts which verified portions of the original structure and also allowed the extension to the new representation were the following:

(1) Ajmaline lost carbon monoxide when heated with Raney nickel in xylene resulting in the formation of a secondary base, decarbonoajmaline. This is undoubtedly best explained as >NCHOHCHEt → >NH + CO + CH_2Et, and in verification of this postulate, decarbonoajmaline gave *n*-butyric acid, less propionic and a trace of acetic acid on oxidation.

(2) Oxidation of ajmaline with permanganate in acetone afforded N-methylisatin thus confirming the presence of >N(a)—CH_3.

(3) The formation of a basic O-dibenzoylajmaline has been confirmed.

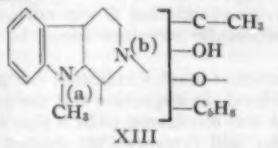
(4) C-Nitrosoajmaline, green prisms, m. p. >330° has now been prepared. This strongly suggests that ajmaline contains no >NH in acid solution. In basic solution, however some of the carbinolamine must be in the form >NH CHO because ajmaline is reduced by borohydride to dihydroajmaline, a secondary base. The hydrobromide of this latter base gave desoxyajmaline hydrobromide on heating. Furthermore, dihydroajmaline could be methylated to dihydro-N-methylajmaline which had been previously obtained (41, 41A) from the lithium aluminum hydride reduction of N-methylajmaline.

(5) When heated with palladium-charcoal catalyst, dihydrodesoxyajmaline [>NH CH$_3$ / CHEt] afforded in small yield a base, $C_{20}H_{24}N_2$, whose ultraviolet absorption spectrum was of the alstyrine (VII, R′ = H, R = C_2H_5) type. On the basis of this new evidence, Robinson and coworkers now represent ajmaline as (XI), in which the asterisked carbon has a tertiary hydroxyl in place of a hydrogen. This unique structure avoids involvement of the tryptamine—CH_2CH_2— side chain in the bridge-ring structure, an undesirable feature of the earlier postulates (X).

(*b*) *Isoajmaline and Neoajmaline.*—These alkaloids were isolated by Siddiqui (11A) from a Dehra Dun variety of *R. serpentina*. Alcoholic potash or heat converted both neoajmaline and ajmaline to isoajmaline. Robinson and coworkers (41, 41A) found that isoajmaline gave derivatives that were similar to those from ajmaline and hence consider the former to be a stereoisomeride of ajmaline. The chemistry of isoajmaline and neoajmaline has not been thoroughly studied as yet and no definite structural conclusions can be drawn at this time.

(*c*) *Rauwolfinine.*—This alkaloid was recently isolated by Chatterjee and Bose (14) from a species of *R. serpentina* collected in the northwestern parts of India. The earlier observations of Siddiqui and Siddiqui (11) that *R. serpentina* specimens grown in different parts of India vary both quantitatively and qualitatively in alkaloidal content were confirmed by Chatterjee and Bose (14) and by Bose (15).

Preliminary investigations (15) showed that rauwolfinine was a monoacidic base containing an N—CH_3, C—CH_2 and two active hydrogens and no methoxy or methylenedioxy groups could be detected. The ultraviolet spectrum (15A, 15B) ($\lambda_{max.}$ 249 mμ, 292 mμ and $\lambda_{min.}$ 226 mμ and 272 mμ) resembled that of ajmaline (15A) and semperflorin (43) and for this reason the authors suggested that rauwolfinine was an indoline derivative. The absorption spectrum also resembled that of yohimbine (44), serpentine (38), and corynantheine (44), but only in the far ultraviolet region and not in the region of longer wavelengths. The infrared spectrum suggested the presence of a tertiary hydroxyl group (2.82μ) an indoline nucleus (intense bands at 6.2μ and 6.8μ), an ether bridge (9.0μ), and a C-methyl group (7.24μ). Absence of bands in the 5.75 to 6μ region nullified the possibility that carboxyl, ester, amide, betaine or carbonyl groups were present. The absorption ascribed to the ether linkage was similar to that observed in the spectrum of serpentine (38, 45). The spectral evidence for the presence of a C-methyl was substantiated by Kühn-Roth determination and the evidence for an indoline structure (XIII) was recently (15B) confirmed by the isolation of Ind-N-methylharman from zinc dust distillation. Alkali fusion of rauwolfinine produced indole-2-carboxylic acid (VI). The nature of a neutral moiety from the zinc dust distillation and a nitrogen-free acid from the alkali fusion is still under investigation. On the basis of the above degradative and spectral evidence Bose (15B) has proposed partial structure (XIII) for rauwolfinine.

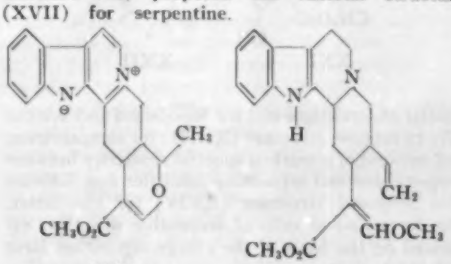


XIII

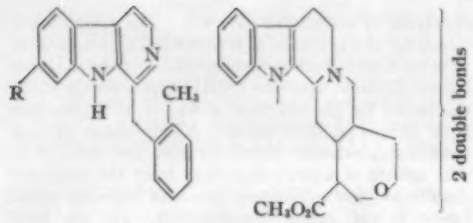
II. **Quaternary Anhydronium bases.**—The term "anhydronium base" was first advanced by Armit and Robinson (46). The structural determination of the Rauwolfia alkaloids in this class was greatly facilitated by the earlier work on harmala bases (47–49), sempervirine (50, 51) and the tetrahydroyohimbines (52).

(*a*) *Serpentine.*—This bright yellow base was first isolated by Siddiqui and Siddiqui (11). They proposed the molecular formula $C_{20}H_{20}O_3N_2 \cdot 1^1/_2$ H_2O, but Schlittler and Schwarz (45), who undertook a more thorough examination of the alkaloid, revised the empirical formula to $C_{21}H_{20}O_3N_2$. The presence of two double bonds that could be hydrogenated, one active hydrogen and one methoxyl group, was readily established. Serpentine was further shown to be an indole alkaloid with a marked similarity to rauwolscine (53–55) and alstonine (36, 38, 56). The presence of an ester group in serpentine was indicated by its infrared spectrum, and was confirmed by the drastic hydrolysis to serpentinic acid, $C_{20}H_{22}O_3N_2$. On the basis of spectral evidence it was suggested that the third oxygen was present as an ether.

The Swiss workers (45) further established the basic character of one of the nitrogens and from spectral studies concluded that no >NH was present. Selenium dehydrogenation of serpentine produced an oxygen-free base alstyrine, $C_{19}H_{22}N_2$ (VII, R = C_2H_5, R′ = H), which had previously been characterized as a dehydrogenation product of alstonine (57, 58) (XIV), and corynantheine (59–61) (XV). Alstyrine and a carbomethoxy group accounted for all of the carbon atoms in serpentine. With the above evidence in hand, Schlittler and Schwarz (45) proposed the skeletal structure (XVII) for serpentine.



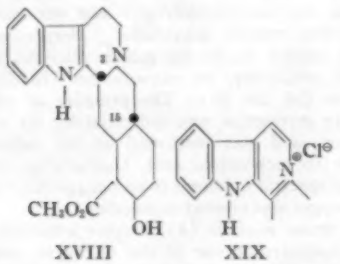
XIV XV



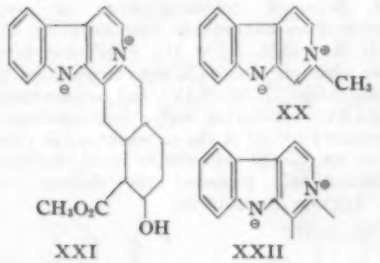
XVI XVII

Since no yobyrine (XVI, R = H) was obtained on dehydrogenation, Ring E was not assigned the normal carbocyclic structure found in yohimbine (XVIII).

Hydrogenation studies indicated the presence of six double bonds in serpentine and the yellow color of the base was considered to be a sign of conjugation. Bader and Schwarz (38) studied the infrared and ultraviolet absorption spectra of serpentine and its derivatives and confirmed this postulate. Moreover, the presence of an absorption band at 6.37μ in the infrared spectrum of serpentine hydrochloride and the absence of this band in *Py*-tetrahydroserpentine was cited as evidence for a conjugated —C=N— group in the hydrochloride and partial structure (XIX) was proposed for it. The ultraviolet spectra of serpentine and its hydrochloride were very similar to those of quaternary-β-carboline (XX) tetradehydroyohimbine (XXI) and their hydrochlorides, indicating the presence of a common chromophore (XXII).

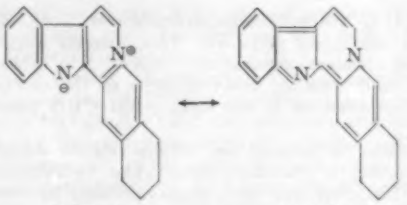


XVIII XIX

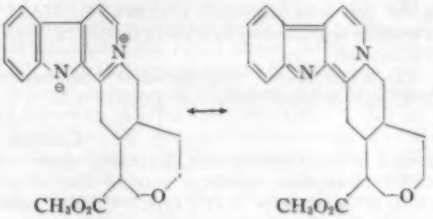


XX XXI XXII

Similar observations had led Woodward and Witkop (50) to propose structure (XXIII) for sempervirine, and because of a marked spectral similarity between sempervirine and serpentine Schlittler and Schwarz (45) proposed structure (XXIV) for the latter. The formation of salts of serpentine was then explained on the basis of the charge separation form (XXIVb).

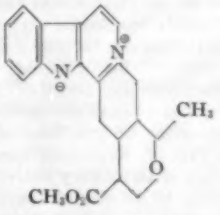


XXIIIb XXIIIa



XXIVb XXIVa

The first revision in the structure (XXIV) for serpentine was made by Bader and Schwarz (38). They proposed that ring E was six membered and contained a C-methyl group, this view being more compatible with the formation of alstyrine (VII, R = C_2H_5, R′ = H) on selenium dehydrogenation (45). The revised structure (XXV) was supposed to be identical with a stereoisomer of the unknown dihydroalstonine and in this connection it was shown that *py*-tetrahydroserpentine and *Py*-tetrahydroalstonine had identical ultraviolet absorption spectra.

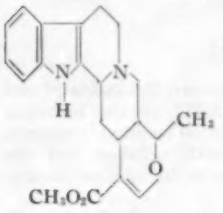


XXV

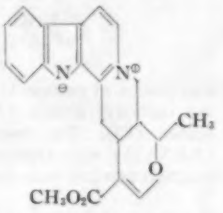
More recently Kloh and co-workers (62) have proposed what is undoubtedly a final revision of the serpentine structure. Analytical data for *py*-tetrahydroserpentine favored a molecular formula differing from that of Bader and Schwarz (38) by two less hydrogens, indicating an additional center of unsaturation. The view was supported by the infrared and ultraviolet absorption spectra of *py*-tetrahydroserpentine which were shown to contain the $H_3COOC-\overset{|}{C}=\overset{|}{C}-O-$ chromophore. The spectral characteristics of this group had been reported by Bader (63) and by Goutarel (64) in their work on alstonine (XIV) and corynantheine (XV) respectively. In addition to this, *py*-tetrahydroserpentine gave the corresponding alcohol, $C_{20}H_{22}O_2N_2$, on lithium aluminum hydride reduction, and the infrared spectrum indicated the presence of a hydroxyl group and an enol ether. The double bond absorption maximum had shifted from 6.2μ

in *py*-tetrahydroserpentine to 6.04μ because it was no longer in conjugation with an ester.

Klohs and co-workers (62) consequently have revised the molecular formula of *py*-tetrahydroserpentine and serpentine to $C_{21}H_{24}O_3N_2$ and $C_{21}H_{20}O_3N_2$ respectively and have proposed structures (XXVI) and (XXVII) for these compounds so that serpentine is in reality a stereoisomer of alstonine.



XXVI



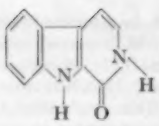
XXVII

(*b*) *Serpentinine.*—This alkaloid, accompanied by serpentine, was first isolated by Siddiqui and Siddiqui (11) and recently by Schlittler and co-workers (65). The Indian workers prepared a series of salts of serpentinine and from their analyses proposed the molecular formula $C_{22}H_{26}O_3N_2$ for the free base. In spite of great analytical difficulties Schlittler, *et al.* (65), have proposed the molecular formula $C_{21}H_{22}O_3N_2$ or $C_{21}H_{20}O_3N_2$ for serpentinine.

The infrared spectrum of serpentinine possessed two bands at 5.83 and 6.16μ that were similar to those found in alstonine at 5.88 and 6.11μ and in serpentine at 5.89 and 6.21μ, thus indicating the presence of the $RO_2CC{=}C{-}O{-}$ chromophore. In contrast to serpentine, however, the presence of a strong band at 2.99μ suggested an alcohol or >NH group. Moreover, several of the characteristic bands (6.38 and 6.28μ, for example) of serpentine (38) could not be detected in the serpentinine spectrum. The ultraviolet absorption spectrum of serpentinine was characteristic of both an indole and a quaternary β-carboline and Schlittler, *et al.* (65), have proposed that it is a base of the anhydronium type.

The presence in serpentinine of two active hydrogen atoms (one probably due to solvent of crystallization) and of a C-methyl group was also reported (65). Serpentinine and serpentine both gave alstyrine (VII, R = C_2H_5, R′ = H) on selenium dehydrogenation. This base accounted for nineteen carbon atoms and the remaining two were presumed to be present as a carbomethoxy group. This ester was much more difficult to hydrolyze than that in serpentine but could be reduced with sodium and butyl alcohol to a compound of molecular formula $C_{20}H_{26}O_2N_2$. The properties of this latter alcohol were similar to those of hexahydroserpentinol (62, 66).

Hydrogenation of serpentinine over platinum failed in basic solution and in acetic acid it was slow and irregular, although a compound was obtained that was similar to *Bz*-tetrahydroserpentine in melting point and ultraviolet absorption spectrum. On alkaline fusion, serpentinine yielded indole-2-carboxylic acid (VI) and a compound that was shown to be identical to pyrid-3-4b-Indole-1(2)-one (67) (XXVIII). Similar treatment of alstonine resulted in the formation of harman (56).



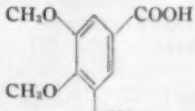
XXVIII

Schlittler and co-workers (65) could not postulate a reasonable structure for serpentinine from the evidence cited above and even the molecular formula remains somewhat obscure at the present time.

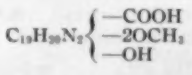
III. **Tertiary Indole Bases of the Yohimbine Type.**—The alkaloids in this group are reserpine, methyl reserpate, rescinnamine (reserpinine), deserpidine, yohimbine, isorauhimbine, serpine, sarpagine (raupine), "Alkaloid 3078," and rauhimbine.

(*a*) *Reserpine:*—The isolation of this base, which is pharmacologically the most important Rauwolfia alkaloid, was first reported by Müller, Schlittler, and Bein (12). Recently Steenhauer (91) has claimed that the alkaloid B reported earlier (13) is identical to reserpine. Reserpine has also been isolated from *R. heterophylla* Roem and Schult by Djerassi and co-workers (68) from *R. canescens* Linn by Klohs and his collaborators (69), from *R. hirsuta* by Vergara (175), from *R. micrantha* by Rao and Rao (181), and from *R. vomitoria* by Janot's group (184).

Reserpine has the molecular formula $C_{33}H_{40}O_9N_2$ and contains six methoxyl groups. The ultraviolet spectrum was quite different from that of other indoles suggesting that if an indole nucleus was present at all it must be a substituted one. The high oxygen content and the presence of a broad band in the ester region made it likely that reserpine was an ester alkaloid (70). Schlittler and co-workers (70, 71) and Neuss, *et al.* (26a, b), independently obtained 3,4,5-trimethoxybenzoic acid (XXIX) and reserpic acid, $C_{22}H_{28}O_5N_2$ (XXX) on alkaline saponification of reserpine. Klohs, *et al.* (72), originally assigned to reserpine the molecular formula $C_{35}H_{44}O_{10}N_2$ and named the hydrolysis product reserpinolic acid. Subsequent work (73) however, confirmed the molecular formula proposed by Schlittler and co-workers (70, 71) and the name reserpic acid has now been adopted for the nitrogenous saponification product.



XXIX

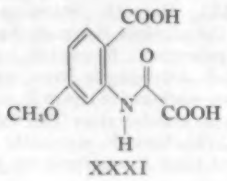


XXX

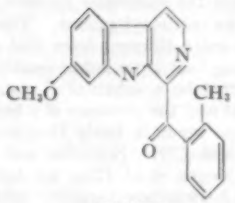
The chemistry of reserpine and reserpic acid was extensively investigated by the Ciba group (70, 71, 73, 74, 74A) and a total structure was proposed on the basis of the following facts. When treated with trimethoxybenzoyl chloride, methyl reserpate yielded a product identical to reserpine. Reserpic acid was shown to contain two methoxyl groups and the presence of a hydroxyl group was strongly indicated by the infrared spectrum. Acylation attempts, however, resulted in the formation of a γ-lactone. If reserpic acid was first esterified with diazomethane, methyl reserpate was obtained and the

hydroxyl group in the ester could be readily acylated or arylated. When reserpic acid was oxidized with permanganate (71), N-carboxyformyl-4-methoxyanthranilic acid (XXXI) was isolated as its dimethyl ester. This established the presence of a methoxyindole moiety in reserpine. Spectral evidence indicated that reserpic acid contained a monomethoxylated tetrahydro-β-carboline system and the assumption was substantiated by a positive Adamkewicz color test (74, 75).

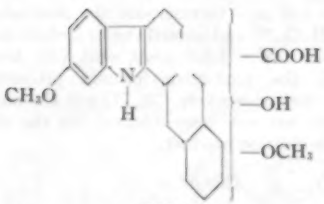
Reserpic acid yielded yobyrine (XVI, R = H) and 7-hydroxyyobyrine (XVI, R = OH) on selenium dehydrogenation. The structure of this yobyrine derivative was confirmed by selenium dioxide oxidation of its methyl ether to the ketone (XXXII) which was in turn compared with a synthetic sample. Reserpic acid was thus shown to contain a pentacyclic ring system and the partial structure was expanded to (XXXIII).



XXXI

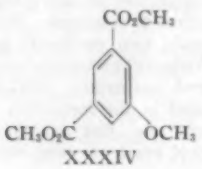


XXXII

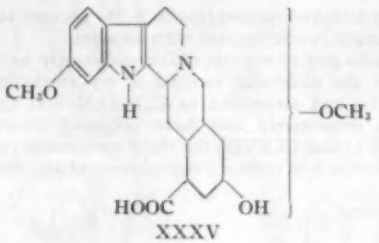


XXXIII

The potassium hydroxide fusion of reserpic acid resulted in the formation of 5-hydroxyisophthalic acid which was isolated as its monomethyl ether dimethyl ester (XXXIV). This degradation product as well as the lactone formed in acylation experiments were best explained by structure (XXXV) for reserpic acid. The remaining methoxyl group

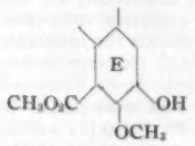


XXXIV

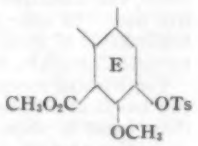


XXXV

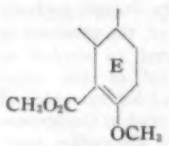
was placed at carbon 17 between the hydroxyl and the carboxyl groups (XXXVI) for the following reason (73). The tosylate of methyl reserpate (XXXVII) was treated with collidine and the reaction product was shown by its infrared absorption to contain the group $RO_2C-\overset{|}{C}=\overset{|}{C}-O$ (5.89 and 6.21μ); the ultraviolet absorption spectrum was in agreement with this conclusion. The structure of the detosylation product (XXXVIII) was further confirmed by the simultaneous acid hydrolysis and decarboxylation to the ketone, reserpone (XXXIX). From the degradations cited



XXXVI



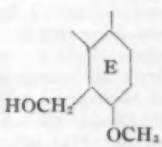
XXXVII



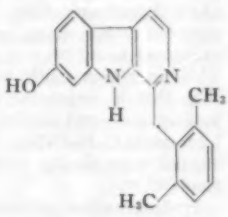
XXXVIII

XXXIX

above Schlittler, *et al.* (74), could safely assume that all three substituents in Ring E were on adjoining carbons but the position of at least one of these groups had to be located exactly before the total structure of reserpine could be established. To this end the tosylate of methyl reserpate (XXXVII) was reduced (74A) with lithium alumi-

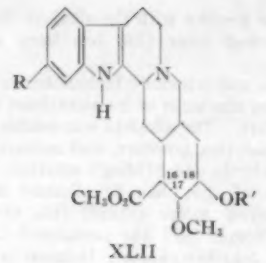


XL

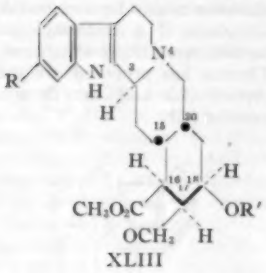


XLI

num hydride to reserpinol (XL) which in turn was dehydrogenated with selenium to give the methyl hydroxyyobyrine (XLI). In this way the carboxyl in reserpic acid was retained in the dehydrogenation product as a methyl group, the location of which was proved by the synthesis of the methyl ether of (XLI). This degradation placed the car-



XLII



XLIII

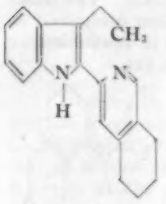
boxyl in reserpic acid at C_{16} and the total structure of reserpine could then be formulated as (XLII) (R = methoxy, R′ = 3,4,5-trimethoxybenzoyl).

(*b*) *Methyl Reserpate (XLII) (R = methoxy, R′ = H).*—The alkaloid was recently isolated from *R. serpentina* by Hofmann (20), who established its identity with the compound obtained from reserpic acid and methanol. It was further shown that methyl reserpate occurred as such in *R. serpentina* and was not a hydrolysis product of reserpine, since the latter was stable under the isolation conditions.

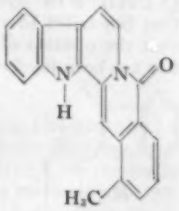
(*c*) *Rescinnamine.*—This base was recently isolated by Klohs, *et al.* (24), and shown by hydrolysis and spectral studies to be the 3,4,5-trimethoxycinnamic acid ester of methyl reserpate (XLII) (R = methoxy, R′ = trimethoxycinnamoyl). Independently, Haack, *et al.* (23), isolated the same alkaloid and gave it the name "reserpinine." "Reserpinine" was also the name given to a C_{22}-$H_{26}O_4N_2$ alkaloid (LIII, R = H) isolated by Schlittler, *et al.* (22). To avoid confusion, it is proposed (180) that the name rescinnamine be retained for the $C_{35}H_{42}O_9N_2$ alkaloid of Klohs and that reserpinine be reserved for Schlittler's C_{22} alkaloid.

(*d*) *Deserpidine:*[2]—Schlittler and coworkers (77B) have reported this base as a minor constituent of many Rauwolfia species including *R. serpentina* and have noted its similarity to reserpine both biologically and chemically. In a recent communication (77A), this similarity has been confirmed by the conversion of methyl deserpidate tosylate (XLIII) (R = H, R′ = $C_7H_7SO_2$—) to α-yohimbine [C_{16} epimer of (XVIII)]. They also proved that epimerization of C_3 occurred in the process so that deserpidine (XLIII) (R = H, R′ = 3,4,5-trimethoxybenzoyl) is a derivative of C_3-epi-α-yohimbine (83).

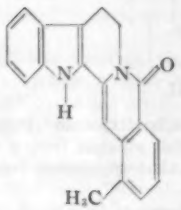
The formation of a γ-lactone from both reserpic acid and deserpidic acid (the acid corresponding to (XLIII) R = R′ = H)), together with the results (unpublished) of elimination reactions, indicate that all three groups in ring E of reserpine and deserpidine are cis. Hence (XLIII) (R = methoxy, R′ = 3,4,5-trimethoxybenzoyl) and (XLIII) (R = H, R′ = 3,4,5-trimethoxybenzoyl) are considered (77A) to represent the complete configuration of reserpine and deserpidine respectively.



XLIV



XLV



XLVI

The Squibb group have recently (182) confirmed these stereochemical assignments for C_{15}, C_{16}, C_{18}, and C_{20} in reserpine by the isolation of a quaternary tosylate containing a bond between N_4 and C_{18} (see XLIII). The formation of this compound by a concerted displacement mechanism requires a cis ring juncture at C_{15}—C_{20} and a β-tosyloxy group at C_{18}. Moreover, molecular rotation differences indicate (182) that (XLIII) (R = methoxy, R′ = 3,4,5-trimethoxybenzoyl) represents the absolute configuration of reserpine.[3]

(*e*) *Yohimbine.*—The isolation of yohimbine (XVIII) has been independently reported by Bader, *et al.*, (77), and by Hofmann (20). The identity of this alkaloid was shown by mixing melting point determination with an authentic sample of yohimbine and its hydrochloride and by comparison of infrared and ultraviolet absorption spectra.

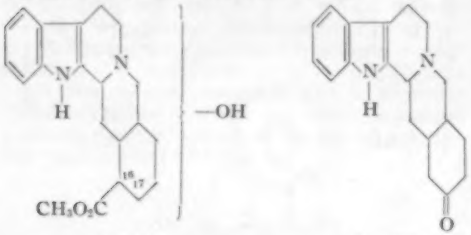
(*f*) *Isorauhimbine.*—This alkaloid was isolated from *R. serpentina* by Hofmann (25) and preliminary work (20, 25) indicated the molecular formula $C_{21}H_{26}O_3N_2$. The presence of two active hydrogen atoms and one methoxyl group was also established.

Further investigations (20, 78) on the constitution of isorauhimbine have indicated that it is probably an isomer of yohimbine (XVIII). Thus, the ultraviolet spectrum was characteristic of the indole chromophore and alkaline hydrolysis yielded isorauhimbic acid which regenerated isorauhimbine on esterification. The selenium dehydrogenation of isorauhimbine produced yobyrine (XVI, R = H), tetrabyrine (XLIV) and dehydroketobyrine (XLV). Yohimbine and its stereoisomers give compounds (XVI), (XLIV), and ketobyrine (XLVI) on selenium dehydrogenation (79, 80). Although a satisfactory explanation for the formation of (XLV)

[2] The same alkaloid (canescine) has been isolated from *R. canescens* by Stoll and Hofmann (174), Klohs, *et al.*, (257) and by the Lilly group (253a).

[3] This stereochemical representation for the reserpine molecule has recently been questioned (273) especially as to the configuration at C_3 (see XLIII).

has not been advanced, these dehydrogenation products establish the pentacyclic nature of the ring system as well as the location of the carbomethoxy group at C_{16}. Consequently, isorauhimbine can be represented by structure (XLVII). The oxidative decarboxylation of (XLVII) would be expected to yield a stereoisomer of yohimbone (XLVIII) if the hydroxyl were at C_{17}. This reaction has failed to yield a crystalline compound so that the position of the hydroxyl in isorauhimbine has not been finally established as yet.



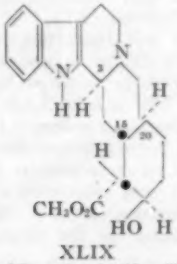
XLVII XLVIII

(*g*) *Serpine.*—Chatterjee and Bose (27) have recently isolated this alkaloid from a Cochin variety of *R. serpentina* that apparently contains no ajmaline.

Serpine was shown to be a weak, monoacidic tertiary base of empirical formula $C_{21}H_{26}O_3N_2$. Tests for the presence of a C-methyl, N-methyl, and methylenedioxy groups were negative. Sulfuric acid produced the characteristic color reaction of tetrahydro-β-carbolines and yohimbine.

The ultraviolet absorption spectrum was similar to that of yohimbine (44) and rauwolscine (55) with maxima at 227, 283, and 290 mμ. The infrared spectrum showed the characteristic absorption bands of an alcohol at 2.75 μ, an imino group at 2.95 μ, and an ester at 5.8 μ. When dehydrogenated in the presence of selenium, serpine produced yobyrine (XVI, R = H), tetrabyrine (XLIV), and ketoyobyrine (XLVI). These products served to establish the pentacyclic nature of serpine and the formation of ketoyobyrine located the carbomethoxy group at C_{16} as in yohimbine. The characteristic Oppenauer oxidation (81–83) product placed the hydroxyl at C_{17} so that serpine becomes a stereoisomer of yohimbine.

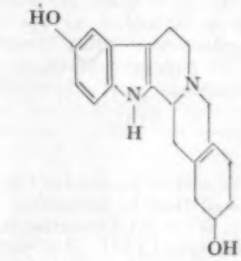
On the basis of experiments, the details of which were not disclosed, the authors further suggested that the carbomethoxy and hydroxyl groups were both axial and that the configuration in serpine (XLIX) at C_3, C_{15}, and C_{20} is the same as in ψ-yohimbine (81).



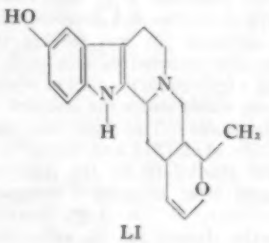
XLIX

(*h*) *Sarpagine* [*Raupine* (*18*)].—This alkaloid has recently been isolated by Stoll and Hofmann (17) and its identity with the alkaloid "raupine" of Bodendorf and Eder (18) has been established (18A).

Sarpagine was originally formulated as a 5-methoxyindole on the basis of its ultraviolet absorption spectrum (84). The alkaloid was soluble in sodium hydroxide solution however, and reduced ammoniacal silver nitrate and Fehling's solution, a behavior reminiscent of the phenolic alkaloid akuammine from *Picralima nitida* (Stapf) (85, 86). Consequently, Thomas (87) has postulated that sarpagine is a 5-hydroxyindole, because a complete yohimbine skeleton cannot be accommodated in the formula $C_{19}H_{22}O_3N_2$ if a methoxyl group is included. The two more likely structures for sarpagine that Thomas has proposed are the hydroxydehydroyohimbol (L) and the decarbomethoxyhydroxyserpentine (LI).



L



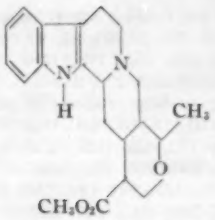
LI

(*i*) *Alkaloid 3078.*—This alkaloid has recently been isolated by Schlittler and coworkers (22A) and preliminary work suggests it to be an isomer of yohimbine.

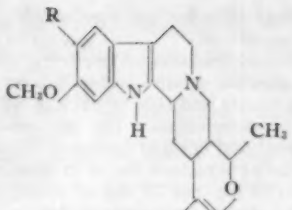
(*j*) *Rauhimbine* (corynanthine).—This base was isolated from *R. serpentina* by Hofmann (25) who later (20) established its identity with corynanthine, the C_{16} epimer of yohimbine (XVIII).

IV. **Tertiary Indole Bases of the Tetrahydroalstonine Type.**—(*a*) *Ajmalicine* (11) (*Py*-tetrahydroserpentine (62), δ-yohimbine (20, 21), alkaloid II (19), raubasine (23), alkaloid F (26)): Ajmalicine was first isolated by Siddiqui and Siddiqui (11) but no molecular formula was suggested for the compound. Klohs and co-workers (62) have recently isolated an alkaloid identical to the ajmalicine of Siddiqui and Siddiqui and to which they assigned the molecular formula $C_{21}H_{24}O_3N_2$. The infrared and ultraviolet spectra were found (62) to be the same as those of *Py*-tetrahydroserpentine as reported by Bader (63) and by Bader and Schwarz (38). The melting points and optical rotations were also found to be in good agreement.

Bader and Schwarz (38) had proposed structural formula (LII) for *Py*-tetrahydroserpentine but on



LII



LIII

the basis of spectral evidence discussed under serpentine, Klohs, *et al.* (62), proposed the structure (XXVI) for *Py*-tetrahydroserpentine and hence for ajmalicine.

Weisenborn, *et al.* (21), isolated from *R. serpentina* an alkaloid $C_{21}H_{24}O_3N_2$ which they showed to be identical with δ-yohimbine isolated from commercial yohimbine (88, 89). They independently assigned structure (XXVI) to δ-yohimbine and established its identity with the *Py*-tetrahydroserpentine of Bader and Schwarz (38). Confirmation of this assignment was provided by the lead tetraacetate dehydrogenation of δ-yohimbine to serpentine (XXVII).

Popelak, *et al.* (19), isolated from *R. serpentina* an alkaloid which they initially name "alkaloid II" and later "raubasine" (23). A molecular formula of $C_{21}H_{28}O_3N_2$ was originally suggested and later (90) corrected to $C_{21}H_{24}O_3N_2$. The identity with *Py*-tetrahydroserpentine (XXVI) was confirmed by the lead tetraacetate dehydrogenation of raubasine to serpentine (XXVII).

Hofmann (20) has also reported the isolation of δ-yohimbine from *R. serpentina*.

Neuss, *et al.* (26c) have isolated an alkaloid which was tentatively called "alkaloid F" but subsequent work has shown it to be identical to the ajmalicine (*Py*-tetrahydroserpentine) characterized by Klohs (62, 72).

(*b*) *Reserpinine* (22) [Alkaloid I (19), raubasinine (23), new alkaloid (21), alkaloid C (20), alkaloid A (26c)]: Reserpinine was first isolated by Schlittler, *et al.* (22), as a minor alkaloid of *R. serpentina* that accompanied reserpine. The molecular formula $C_{22}H_{26}O_4N_2$ was assigned and the presence of one C-methyl, two methoxyls and one active hydrogen was demonstrated.

The chemical and spectral evidence indicated that reserpinine was identical with "alkaloid I" (raubasinine) reported by Popelak, *et al.* (19, 23). The absorption maxima recorded for reserpinine were 229 and 298 mμ and for alkaloid I were 228–230 and 298 mμ. The infrared spectrum showed absorption bands at 2.96 (—NH), 5.87 and 6.24 μ (conjugated ester) and 12.05–12.5 μ (1,2,4-trisubstituted benzene ring). From this evidence, coupled with the results of other reactions, details of which are not yet published, Schlittler, *et al.* (22), proposed structure (LIII) (R = H) for reserpinine. Weisenborn, *et al.* (21), isolated a "new alkaloid" from *R. serpentina* and assigned to it the molecular formula $C_{22}H_{26}O_4N_2$. Independently they arrived at structure (LIII) (R = H) for this alkaloid.

The presence of a carbomethoxy group was demonstrated by alkaline hydrolysis followed by the regeneration of the alkaloid when the free acid was allowed to react with diazomethane. The position of second methoxyl group was shown to be C_{11} by comparison of the ultraviolet spectrum with that of methyl reserpate (XLII) (R = methoxy, R′ = H). The presence of the chromophore system $H_3COOC—\overset{|}{C}=\overset{|}{C}—O—$ was indicated by the characteristic infrared absorption at 5.85 and 6.21μ (63).

Hofmann (20) has reported the isolation of a base (alkaloid C) whose melting point, molecular formula and specific rotation are in close agreement with those of reserpinine (22), strongly suggesting their identity.

Neuss, *et al.* (26c), have shown that their "alkaloid A" is identical with Weisenborn's (21) reserpinine.

(*c*) *Reserpiline.*—This amorphous base has been reported recently by the Riker group (24A) as a minor alkaloid of *R. serpentina* and independently, Stoll's group (183) has isolated the same material from *R. canescens*. The molecular formula, $C_{23}H_{28}O_5N_2$, differed from that of reserpinine by the elements CH_2O, corresponding to an extra methoxyl group. This latter group was located in the 5-position of the indole moiety since the ultraviolet absorption spectrum of reserpiline was identical with that of 2,3-dimethyl-5,6-dimethoxyindole.

The infrared spectrum contained the two peaks at 5.99 and 6.20μ which are characteristic (63) of the $RO_2C—\overset{|}{C}=\overset{|}{C}—O—$ chromophore that occurs in ajmalicine, reserpinine and alstonine and the typical shift (62, 72A) of the enol ether band to 6.09μ in the lithium aluminum hydride reduction product was also observed.

Although the small amount of reserpiline available precluded extensive degradative studies, structure (LIII) (R = OCH_3) has been assigned to this base with reasonable certainty (24A, 183).

V. **Alkaloids of Unknown Ring Structure.**—(*a*) *Ajmalinine.*—This alkaloid, $C_{20}H_{26}O_3N_2$, was isolated by Siddiqui and Siddiqui (11), who proved it to be a tertiary base containing a methoxyl and a hydroxyl group. No methylimino group could be detected. When ajmalinine was heated to 200° in an atmosphere of nitrogen, apoajmalinine, $C_{13}H_{17}O_3N_2$, was formed. The ultraviolet absorption spectrum suggests the presence of a 5-methoxyindole moiety (84) but incomplete chemical information negates the possibility of assigning a reasonable structure to this alkaloid.

(*b*) *Serpenine.*—The isolation of this alkaloid was first reported by Chatterjee and Bose (27), and in a recent communication, Bose (16) has elaborated somewhat on the chemistry of this minor constituent. Analysis indicated the formula $C_{20}H_{24}$-

ON_2 or $C_{21}H_{26}ON_2$ for the base which melts and sublimes at 315–317°.

The alkaloid contains no methoxy, methylenedioxy or phenolic groups, feebly reduces ammoniacal silver nitrate and fails to give the typical color reaction of β-carbolines. The ultraviolet ($\lambda_{max.}$ 250, 293 mμ) and infrared spectrum are suggestive of the indoline structure found in ajmaline. The low yield (20 mg. per 75 Kg. of roots) has made degradative studies impossible to date.

(*c*) *Chandrine.*—The isolation of this alkaloid, $C_{23}H_{26}O_4N_2$, has recently been reported but no experimental details were given (179).

(*d*) *A "New Alkaloid" and an Amphoteric Alkaloid.*—These two alkaloids were isolated by Siddiqui and Siddiqui (11) and no evidence except for the melting points was reported. It is likely that these two alkaloids correspond to some of the alkaloids isolated in later years that have been reported under other names.

VI. **Non-indole Alkaloids.**—Hofmann (20) has reported the isolation of two non-indole alkaloids from *R. serpentina* and by mixed melting point determinations coupled with a comparison of ultraviolet and infrared spectra has shown them to be thebaine and papaverine.

PHARMACOLOGY

Because of the prodigious volume of literature concerned with the pharmacology[4] of Rauwolfia serpentina alkaloids, no attempt will be made to discuss the matter in detail, but instead, a few salient characteristics of the drug, its possible mode of action, and its uses in medicine will be described and a substantially complete bibliography on the subject will be supplied for the interested reader.

Prior to the discovery of reserpine (12) the principal alkaloids that had been studied as chemical individuals were ajmaline (10, 11, 76, 93, 112, 129–131, 132e, g, h, 133, 137, 138), iso- and neoajmaline (76, 134), rauwolfinine (14, 137), serpentine (10, 11, 76, 93, 112, 131, 132c, d, g, 137, 138, 170), serpentinine (10, 11, 76, 93, 112, 131, 132f, g, 137, 138), ajmalinine (11, 76, 131, 132a, b), and ajmalicine (132a, 136). An excellent review that clarifies the conflicting results of the early Indian investigators has been published (92). With the possible exception of serpentinine, the above-mentioned alkaloids were found to be hypotensive in nature.

The use of *Rauwolfia serpentina* in hypertension was first reported outside of India by Vakil in 1949 (100) and in the few years since then an almost unprecedented amount of work has been published on the pharmacology of the drug. Because various preparations have been used in these experiments the results are at times confusing. There is general agreement, however, that the powdered root, the alseroxylon fraction, and the pure component reserpine all have hypotensive activity (10, 12, 76, 93–129, 144–150, 153–172, 176, 185–218, 244, 246–249, 258, 259) and the mechanism of this action has been the subject of wide investigation both for reserpine (12, 76, 119, 135, 142–149, 154, 157–159, 165, 167, 168, 189, 192, 204, 207, 210, 215, 217, 219, 221–225, 228, 229, 231, 233, 238, 250, 251, 255, 260–262) and for various other forms of the drug (10, 11, 14, 18, 23, 24, 93–95, 97, 111, 115, 116, 119, 122, 131–136, 152, 173, 188, 193, 195, 202, 216, 220, 226, 227, 230, 232, 252–254, 256, 263).

Another outstanding property of most Rauwolfia preparations (10, 13, 97, 98, 103, 107, 108, 110, 113, 115, 118, 122, 126, 127, 138, 140, 152, 196, 207, 209, 220, 230, 236, 243) and of reserpine especially (12, 76, 139, 141–144, 146, 151, 160, 165, 166, 204, 206, 210, 215, 229, 238, 242, 250) is a marked sedative action on the central nervous system which apparently is independent of the hypotensive activity (190, 204, 217, see however, 210) and is in sharp contrast to the barbiturates in its mode of action (123, 141, 143, 144, 146, 152, 254). The sedation produced by the mixed alkaloids (98, 105, 122, 150, 197, 234–236, 241) and by reserpine (151, 237–240, 264, 269) seems to be of considerable importance in the treatment of various mental disorders and if the long-range results bear out the findings of short-range observations, it is claimed (238) that the drug will represent the most important therapeutic development in the history of psychiatry.[5]

Although Rauwolfia preparations have been used most widely in the treatment of hypertension and mental disorders, various reports indicate that the drug has potentialities in the field of geriatrics (160, 200, 206, 243, 271) and to a lesser extent in the treatment of psoriasis (171, 245), angina (190, 270), constitutional leanness (171), and gynecologic disorders (242).

Rauwolfia serpentina preparations and the pure alkaloid reserpine possess two properties that greatly increase their therapeutic utility. These are an apparent low toxicity (125, 168, 256, 259) and freedom from serious side effects. The most common complaints have been reported by Moore, *et al.* (218), as lethargy and muscular relaxation, drowsiness, nasal congestion or stuffiness, rhinorrhea, increased frequency of bowel movements, diarrhea, dizziness, decreased libido and potentia, tendency to gain weight, nightmares or disturbing dreams, agitated depression and dyspnea at rest. Many of these reactions have been substantiated by other investigators for various forms of the drug (24, 119, 127, 129, 144, 146, 147, 149, 151, 156, 160, 165, 168, 177, 186, 188–190, 194, 196, 197, 200, 206, 211, 215, 217, 249, 252, 259, 270, 271) along with bradycardia (24, 119, 127, 129, 142, 146–151, 156, 168, 190, 206, 211) and miosis (164, 189). Moyer (119) has recently shown that there is very little difference in the incidence of these side reactions when reserpine and Rauwiloid (an alkaloidal extract of *R. serpentina*) are compared. It should be noted that doses above the normal therapeutic level have on occasion caused parkinsonism (105, 215, 239, 266, 269) but the effect disappeared when the drug was discontinued.

The actual mechanism involved in the hypotensive and sedative action of Rauwolfia serpentina preparations is still not clear, although there is general agreement that the effects observed are mainly due to actions within the central nervous system. This has been suggested on the basis of

[4] For reviews on this subject see references 15A, 28, 76, 92, 101, 151, 177, 207, 217, 218, 230, 265.

[5] A symposium on the use of reserpine in the treatment of neuropsychiatric, neurological, and related clinical problems was recently held and the results are now available in published form (274).

experiments with reserpine (12, 28, 119, 135, 142–150, 157, 165–168, 190, 204, 217, 222, 224, 228, 230, 250, 255) and also with other forms of the drug (111, 127, 136, 150, 190, 195, 202, 207, 216, 217, 230, 254, 256). The complex pattern often observed suggests receptors other than those commonly recognized (148) so that a duality of mechanism, including a direct peripheral reaction (142A, 225), is a distinct possibility. It is beyond the scope of this paper to discuss the conflicting pieces of evidence which have recently been reviewed (217, but see 267).

It should be mentioned that until recently, reserpine has been considered the most potent chemical individual in Rauwolfia serpentina preparations. However, two other alkaloids, rescinnamine (23, 24, 173b, 220, 252) and canescine (deserpidine) (77B, 174, 176, 253a, b, 268) produce essentially the same pharmacological effects as does reserpine and results of further tests on these two compounds will be awaited with considerable interest.

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Determination of Reserpine in Pharmaceutical Preparations*

By ROGER E. BOOTH

A method of assay for reserpine in pharmaceutical preparations is described. By this method the small amounts of reserpine usually found in commercially available fluid or dry formulations can be determined. A second step is included to indicate the extent of hydrolysis of reserpine which may occur in the presence of moisture.

THE BROAD USEFULNESS of reserpine in therapeutics has led to its inclusion in a variety of pharmaceutical preparations. Published methods for its determination up to the present time have been limited to its ultraviolet spectrophotometric characteristics (1, 2). One further assay method for reserpine in tablets has been proposed (3) based on a vanillin reagent. Since the dosage level of reserpine is relatively low (0.1–1.0 mg.) most forms for administration contain a very small percentage of active ingredient. Sufficient sample for ultraviolet assay of the dry forms (tablets) can usually be utilized. However fluid forms (i.e., elixirs containing 0.05 mg. per ml.) cannot be assayed in this way. Not only do the flavors and dyes interfere with the ultraviolet spectrum of reserpine, but the extent of possible hydrolysis is not indicated. A precipitation of the reserpine from the elixir has been used (4) but the quantitative precipitation of such small quantities is not feasible with certain vehicles.

This paper describes a method of assay for reserpine in pharmaceutical preparations based on a modification of one for the estimation of tropane alkaloids proposed by Durick and others (5). A second step is used to show the extent of hydrolysis, if any, which may occur in the fluid products.

EXPERIMENTAL

Materials and Method

For Total Reserpine.—*Dye Solution.*—Dissolve 200 mg. of bromphenol blue in 30 ml. of distilled water and 6 ml. of 0.1 *N* sodium hydroxide. Dilute to 250 ml. with water.

Buffer Solution.—Dissolve 10.93 grams of anhydrous disodium hydrogen phosphate and 10.98 grams of anhydrous citric acid in sufficient water to make one liter. The resulting solution should have a *p*H of 4.0.

Standard Reserpine Solution.—Dissolve 25 mg. of reserpine in chloroform and dilute to 200 ml. Take 20 ml. and dilute to 200 ml. with chloroform. The resultant standard solution contains 0.0125 mg. reserpine per ml.

* Received April 1, 1955, from the Upjohn Company, Kalamazoo, Mich.

Chloroform U. S. P.

Disodium Hydrogen Phosphate Solution 10% in Deionized Water.

PROCEDURE

To each of four 125-ml. separators add 20 ml. of buffer and 5 ml. of dye solution. To separator 1 add 5 ml. (0.25 mg. reserpine) of elixir reserpine followed by 50 ml. of chloroform; to separator 2 add 50 ml. of chloroform; to separator 3 add 15 ml. of standard reserpine solution followed by 35 ml. of chloroform; to separator 4 add 25 ml. of standard reserpine solution followed by 25 ml. of chloroform. Shake the mixtures simultaneously for three minutes. Allow the mixtures to stand until separated. Withdraw the chloroform layers into 50-ml. Erlenmeyer flasks. Any remaining emulsion is withdrawn and discarded. Carry out a second extraction of the elixir reserpine sample in the same manner with exactly 50 ml. of chloroform (solution 5). Read the absorbances of these five solutions against chloroform at 402 mμ in 1-cm. cells. Prepare a standard curve from the results of the standards and determine the amount of reserpine in the samples from the curve. The total reserpine in 5 ml. of elixir is equal to the sum determined in the two extractions (solutions 1 and 5).

For Per Cent Hydrolysis.—To 5 ml. of elixir (0.25 mg.) add 10 ml. of a 10% solution of disodium hydrogen phosphate. Extract the resultant solution immediately with chloroform (25, 15, 10 ml., four minutes each). Combine the chloroform and make up to exactly 50 ml. Extract the sample further with chloroform (25, 25 ml., four minutes each) and again collect and make up to 50 ml. Treat these two portions the same as a portion of the standard reserpine solution in the method for total reserpine. The reserpine extracted equals the sum of the two portions. The per cent hydrolysis equals the difference between total reserpine and extracted reserpine in mg./ml. $\times 2 \times 1000$.

DISCUSSION

Standard Curve.—A standard curve is prepared for each run. Different lots of dye and buffer and a variation in the extraction times give slightly different curves. A typical standard curve is given in Fig. 1.

It should be pointed out that a complete separation of the reserpine-dye complex is possible with one extraction when the reserpine is in the chloroform layer. A second extraction merely gives the value of a blank. This is the case for all the standard reserpine solutions and the chloroform-extracted

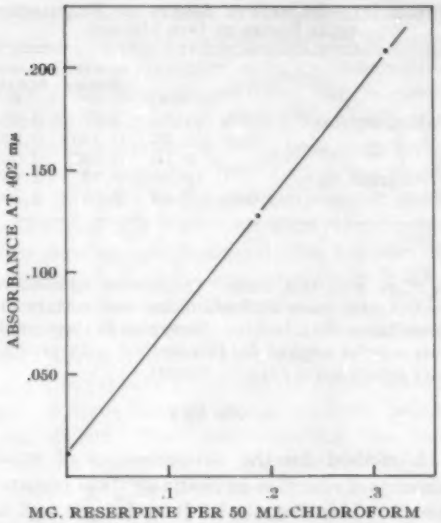

Fig. 1.—Typical standard curve of reserpine-dye complex

samples. However, when the reserpine is in the aqueous phase (as in original elixir) about 85% of the complex is extracted in the first chloroform portion, the remaining 15% in the second portion. A third extraction, again, gives the same value as a blank elixir vehicle. The quantity of uncomplexed dye is in sufficient excess to allow this. Since each of the two extractions is determined as an individual sample there are no concentration errors. The sum of the reserpine found in the two portions is equivalent to the total in the elixir sample as both portions were obtained from the same sample. If desired, the standards and elixir sample may both be extracted twice, the total extractions made up to equal volumes and the readings taken. The numerical readings are reduced to approximately one-half those given in the standard curve (Fig. 1). This may be overcome by running a separate series of standards with each of the two extractions of the elixir. In view of the preceding investigation, the latter two modifications of this method had not been considered necessary.

The standard chloroform solution of reserpine is not stable for extended times. A fresh standard solution is prepared every three days. Even when stored in a dark place, the chloroform solution of reserpine begins to color after this length of time. If the standard solution is assayed when it has become colored, low results (>10%) are obtained compared to a fresh standard solution. This suggests an assayable breakdown of the reserpine nucleus other than hydrolysis.

Effect of Blanks.—Normally a vehicle blank is required for this type of assay. In this case the values obtained on vehicle blanks were insignificantly different than on chloroform alone (Table I). This does not preclude the possibility of obtaining high blanks on future formulations.

Separation of Reserpic Acid.—In the assay for total reserpine, any reserpic acid present will also be determined as reserpine. This is shown by the addition of reserpic acid to the vehicle blank and assaying as for reserpine. About ninety-four per cent recovery was obtained calculated as reserpine. Addition of reserpic acid to the vehicle blank and assaying as described in the hydrolysis step for reserpine gave 0% recovery. Actual hydrolysis of reserpine with alkali and then assaying as described gave 0% recovery. Thus the difference between the assay for *total* reserpine and *recovered* reserpine is equivalent to the reserpic acid present (% hydrolysis).

TABLE I.—COMPARATIVE ABSORBANCES OF VEHICLE BLANKS AND CHLOROFORM BLANKS

Sample	Absorbance	
	Elixir Blank	Chloroform Blank
1	0.012	0.010
2	0.016	0.011
3	0.016	0.012
4	0.008	0.010
5	0.009	0.011
6	0.011	0.008

Reproducibility.—The ability to duplicate results is shown in Table II. Reproducibility is dependent at least in part on the solubility of the dye-reserpine complex in chloroform.

TABLE II.—RECOVERY OF RESERPINE FROM ELIXIR

Theory	Found	
	Sample A	Sample B
0.050 mg./ml.	0.050	0.049
	0.049	0.048
	0.051	0.050
	0.050	0.052
Mean	0.050 mg./ml.	0.050 mg./ml.

Since two extractions are necessary to remove the complex completely the errors are somewhat greater than if a total extraction could be accomplished with one portion of chloroform.

Nature of the Method.—Bromphenol blue is insoluble in chloroform. When combined with reserpine a complex is formed which is chloroform-soluble and thereby extracted. The dye reacts with reserpine in the molecular ratio of 1:2. This was shown by titration of a known quantity of reserpine with a standard dye solution in *p*H 4 buffer. After each increment of dye was added, the mixture was thoroughly shaken. The end point was indicated by an increase of dye color in the aqueous layer. A further test confirmed this titration. Equal known quantities of reserpine were placed in each of four flasks. Buffer was added followed by dye in one to four equivalents. After standing with occasional shaking for twenty-four hours, the residual dye was observed. The samples containing a ratio of 1:1 and 3:4 showed a large excess of dye remaining; that of 1:4 showed insufficient dye to react; that of 1:2 showed a very slight excess (comparable to the end point in the above titration procedure). This information correlates well with the structure suggested by Auerbach (6) for the reaction of tetrabromophenolsulfonphthalein with quaternary ammonium compounds.

The influence of *p*H in this method is marked. The preferred *p*H is that of the transition range of

the dye. In all cases uniformity of *p*H is essential and more important than the absolute *p*H value within a narrow range. Accordingly sufficient buffer must be used to establish the desired *p*H.

Applications.—In addition to elixirs the method herein described has been utilized satisfactorily on various commercially available dosage forms of reserpine: tablets (0.1, 0.25, 1.0 mg.), triturations (2.5% with lactose) and sterile solutions (2.5 mg./ml.). The treatment of each of these forms prior to the formation of the alkaloid-dye complex follows:

Tablets.—Reduce a representative number of tablets to a fine powder. Place a weighed portion equivalent to 0.50 mg. of reserpine in a separator containing 10 ml. of water. Shake vigorously for five minutes. Add 10 ml. of disodium hydrogen phosphate solution and extract immediately with four 25-ml. portions of chloroform. Combine the chloroform extracts and make up to exactly 100 ml. Take an aliquot of this chloroform solution equivalent to 0.15–0.25 mg. reserpine and treat exactly as the standard reserpine solutions in the method for total reserpine.

Triturations.—Place a weighed portion of trituration equivalent to 2.50 mg. of reserpine in a separator containing 10 ml. of water. Complete as described under *Tablets* making the total chloroform extracts up to 500 ml.

Sterile Solution.—Dilute the sample with water to give 2.50 mg. reserpine in 50 ml. Take exactly 10 ml. of diluted sample and complete as described under *Tablets* beginning with, "Add 10 ml. of disodium hydrogen phosphate solution . . . . "

Typical assay results on these dosage forms are given in Table III:

TABLE III.—RESERPINE ASSAYS OF PHARMACEUTICAL FORMS BY DYE METHOD

	Theory	Found Sample A	Found Sample B
Tablet, mg./tab.	1.0	0.97	0.98
	0.25	0.250	0.250
	0.10	0.095	0.095
Trituration, %	2.50	2.45	2.48
Sterile Solution, mg./ml.	2.50	2.54	2.58

In general it has been found that barbiturates, steroids, organic acids, and certain amines do not interfere with this assay. Quaternary ammonium compounds, some antihistamines, and certain organic bases do interfere. Reserpine in those products may be assayed by this method with preliminary separation or *p*H adjustments.

SUMMARY

A method for the determination of small amounts of reserpine in pharmaceutical preparations has been described. It lends itself well to both the dry and fluid dosage forms of reserpine commercially available. By this method, the extent of hydrolysis of reserpine which may occur in the presence of moisture can be determined.

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Adsorption of Some Alkaloids by Different Clays*

By NUR EVCIM and MARTIN BARR

The adsorption of strychnine, atropine, and quinine from aqueous solution by six different clays was studied. The clays were Permagel, attapulgite, halloysite, dickite, kaolinite, and kaolin N. F. Adsorption isotherms at 24 ± 1° were determined and Langmuir's constants were calculated. The adsorption power of the clays was found to be in the same order for each of the three alkaloids. Permagel, attapulgite, and halloysite were better adsorbents for the alkaloids than kaolin N. F. Dickite was found to be a poor adsorbent. The effect of *p*H on the adsorption was studied in one of the cases.

THIS PAPER PRESENTS a preliminary study of the adsorptive properties of five different clays, considering their possible medicinal and toxicological uses. Kaolin N. F. IX was included in the investigations for comparative purposes.

The clays studied have been reported in the geological literature. These are attapulgite, halloysite, dickite, kaolinite, and Permagel,[1] a purified form of attapulgite. These clays were obtained[2] in rock-like pieces and finely powdered[3] before investigation.

Attapulgite is a hydrous magnesium aluminum silicate in the palygorskite group. Its ideal formula was assigned by Bradley (1) as $(OH_2)_4(OH)_2Mg_5Si_8O_{20}.4H_2O$. Permagel is a highly

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[1] Attapulgus Minerals and Chemicals Corporation, Philadelphia, Pa.
[2] Samples were obtained through Ward Natural Science Establishment, Rochester, N. Y.
[3] Bantam Type W Mikro-Pulverizer used for pulverization at 14,000 r.p.m. with a 0.010" cross slot screen.

colloidal, purified form of attapulgite. Halloysite is a clay of composition near that of kaolinite. There is confusion as to its nomenclature since it occurs in different forms, but the general name halloysite with modifying names indicating degree of hydration, etc. has been suggested as the best nomenclature (2). Dickite is a mineral from the Island of Anglsey (3) and originally called kaolinite. Ross and Kerr (4) recognized this same material in several other localities and ascertained that it was structurally distinct from other species in the kaolin group. Kaolinite is the mineral characteristic of kaolin having the general formula $Al_2O_3.2SiO_2.2H_2O$. Ross and Kerr (4) have shown that the kaolin minerals are of three distinct species, kaolinite, nacrite, and dickite. They suggested that the name kaolinite be kept for the characteristic mineral of kaolin. The name of the official clay, kaolin, is, therefore, generic rather than specific. Mineralogically, the term is used as a group name for at least four separate minerals: kaolinite, nacrite, dickite, and anauxite.

Average and maximum particle sizes of the clay samples are recorded in Table I. Chemical composition of the clay samples is shown in Table II.

TABLE I.—PARTICLE SIZES OF CLAY SAMPLES[a]

Name	Av. Part. Size	Max. Part. Size
Permagel	[b]	[b]
Attapulgite	10 μ	75 μ
Halloysite	10 μ	75 μ
Kaolinite	5 μ	50 μ
Dickite	10 μ	75 μ
Kaolin N. F.	5 μ	45 μ

[a] Determined by Arthur L. Stern Company, South Orange, N. J.
[b] 100% passes through a 325-mesh sieve.

TABLE II.—CHEMICAL COMPOSITION OF CLAY SAMPLES

	Attapulgite,[a] %	Halloysite,[a] %	Dickite,[a] %	Kaolinite,[a] %	Permagel[b] %
SiO_2	54.04	44.46	40.80	45.11	42.26
Al_2O_3	9.83	36.58	35.70	37.02	8.50
Fe_2O_3	3.52	0.36	...	1.24	3.20
FeO	0.19	0.07	0.04	0.17	...
MgO	9.87	0.18	trace	0.11	7.00
CaO	1.69	0.19	3.96	0.22	1.40
Na_2O	0.08	0.01	0.20	0.36	2.00
K_2O	0.57	0.51	0.20	0.71	
P_2O_5	...	0.18	...	...	...
TiO_2	0.32	0.15	0.02	1.30	0.30
H_2O	10.93	13.38	17.68	13.44	35.00
H_2O	10.00	4.05	1.95	0.80	
	100.24	100.12	100.55	100.48	100.00

[a] As reported by American Petroleum Institute, Project 49, Preliminary Report No. 7, Columbia University, N. Y.
[b] Bulletin No. P53, p. 3, Attapulgus Minerals and Chemicals Corp., Philadelphia, Pa.

EXPERIMENTAL

Adsorption isotherms at room temperature (24 ± 1°) for strychnine, atropine, and quinine were determined in the following manner.

A standard alkaloid solution was prepared by dissolving an appropriate amount of the alkaloidal sulfate in distilled water. The concentration of the solution was determined spectrophotometrically or colorimetrically, with the methods described later for assaying the samples after adsorption. For clays with a relatively high adsorption capacity, a solution containing approximately 2 mg. alkaloid base per cc. was used; for those with low adsorption capacity, approximately 0.5 mg. alkaloid base per cc. With quinine, only the latter could be used, because of the low solubility of quinine sulfate.

An accurate volume of the standard alkaloid solution was measured into a 100-cc. volumetric flask from a buret and was diluted to volume with distilled water. This solution was transferred into a 200-cc. container and 0.50 Gm. of clay was added. The container was closed and shaken continuously for thirty minutes. (It was determined that equilibrium conditions existed after thirty minutes of shaking.) At the end of this period, portions of the mixture were centrifuged and the clear supernatant fluid was taken for analysis. The amount of alkaloid base adsorbed by the clay was calculated by difference.

Determination of Strychnine and Quinine.—The spectrophotometric method of Bhattacharya and Ganguly (5) and Biggs (6) was used for the determination of strychnine. The spectrophotometric method reported by Carol (7) was used for the determination of quinine. The ultraviolet absorption curves for both alkaloids were determined before employing the methods. The wave lengths of maximum absorption and the corresponding values of $E^{1\%}_{1\,cm.}$ were found to be in agreement with the references. The wavelength used for strychnine was 255 mμ ($E^{1\%}_{1\,cm.}$ = 377) and for quinine 347.5 mμ ($E^{1\%}_{1\,cm}$ = 168).

Following the centrifugation, an aliquot of the clear supernatant liquid was transferred into a volumetric flask, acidified with sulfuric acid and diluted with water so that a solution was obtained in which the concentration of the alkaloid was within the optimum range for spectrophotometric analysis. Acidification of the solution to approximately 0.1 *N* with sulfuric acid repressed hydrolysis of the alkaloidal salt and insured stable *p*H of the solutions. A Beckman Model DU Spectrophotometer with 1-cm. silica cells was employed for the determinations. The blank used was 0.1 *N* H_2SO_4.

Determination of Atropine.—The colorimetric method described by Bandelin (8), and Colby and Beal (9) was used to determine atropine. The Beckman Model DU spectrophotometer was employed also for this assay.

The isotherms were determined by repeating the described procedure with at least five different alkaloid concentrations, each in duplicate, for every alkaloid-clay combination (10). Milligrams of alkaloid base adsorbed per Gm. clay were plotted against the equilibrium concentration measured in mg. per 100 cc. Figures 1, 2, and 3 show the adsorption isotherms for strychnine, atropine, and quinine, respectively.

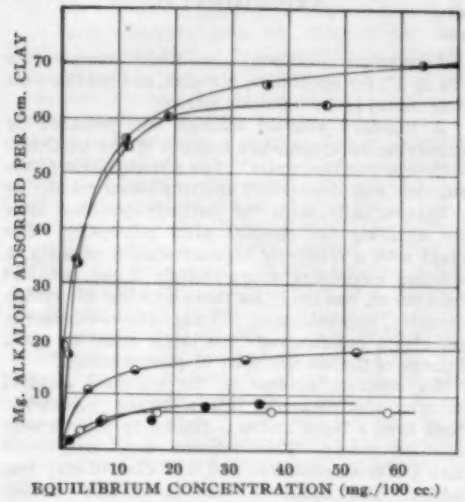

Fig. 1.—Adsorption isotherms at 24° C. for strychnine by clays. —●— kaolin, —○— kaolinite, —◒— halloysite, —◐— attapulgite, —◑— Permagel.

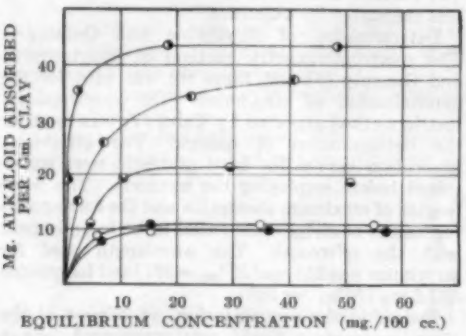

Fig. 2.—Adsorption isotherms at 24° C. for atropine by clays. —●— kaolin, —○— kaolinite, —◒— halloysite, —◐— attapulgite, —◑— Permagel.

DISCUSSION

Methods that have previously been described (11, 12) in the pharmaceutical literature for the evaluation of the adsorptive power of adsorbents for alkaloids fail to give a true picture of the adsorption phenomena. The adsorption isotherm shows the effect of concentration on the amount adsorbed from solution and also provides a suitable way for the comparison of the adsorptive power of different adsorbents at different concentrations.

As seen in the figures, the adsorptive power of the clays was in the same order for each of the three alkaloids. Permagel, attapulgite, and halloysite were better adsorbents for alkaloids than kaolin N. F. IX. The adsorptive capacity of kaolinite was about equal to that of kaolin. Dickite was found to be a very poor adsorbent for alkaloids, since in no case was the adsorption of the alkaloid

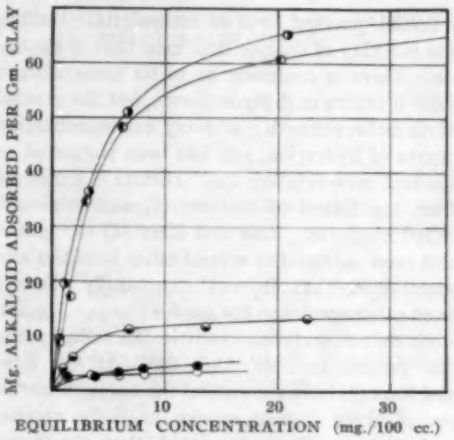

Fig. 3.—Adsorption isotherms at 24° C. for quinine by clays. —●— kaolin, —○— kaolinite, —◒— halloysite, —◐— attapulgite, —◑— Permagel.

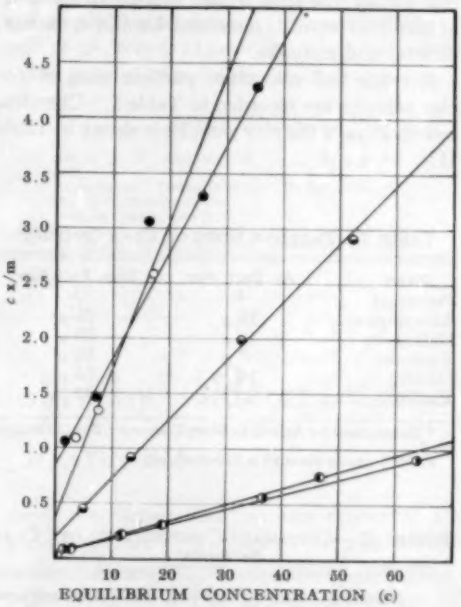

Fig. 4.—Linear form of the adsorption isotherms at 24° C. for strychnine by clays. —●— kaolin, —○— kaolinite, —◒— halloysite, —◑— attapulgite, —◑— Permagel.

base higher than 2 mg. per Gm. dickite. Therefore, isotherms were not plotted for this clay.

The curves show that the adsorption reaches a saturation point at high equilibrium concentrations. Such isotherms are best represented by the Langmuir equation (10):

$$\frac{x}{m} = \frac{abc}{1 + ac}$$

where

$\frac{x}{m}$ = mg. alkaloid base adsorbed per Gm. of adsorbent,

c = equilibrium concentration in mg. alkaloid base per 100 cc., a and b are constants.

From the equation it can be seen that at high equilibrium concentrations the value of x/m approaches that of the constant b. Therefore, the value of the constant b is a good measure of the adsorption capacity of adsorbents. The constant b is expressed in terms of mg. alkaloid base adsorbed per gram of adsorbent.

The linear form of the Langmuir equation, derived from the equation given above, is:

$$\frac{c}{x/m} = \frac{1}{ab} + \frac{c}{b}$$

The applicability of the Langmuir equation to the isotherms can be indicated by the linear curves obtained on plotting $\frac{c}{x/m}$ against c. As an example, Fig. 4 shows the linear curves obtained for strychnine with clays.

The constants a and b of Langmuir's equation were calculated for each alkaloid-clay combination by plotting the linear curves corresponding to Figs. 1-3 on a large scale in order to obtain the slope of the lines $[1/ab\ (c)]$ and their y-intercepts $(1/b)$ more accurately. These values are reported in Table III.

TABLE III.—CONSTANTS OF LANGMUIR'S EQUATION

Clay	For Strychnine		For Atropine		For Quinine	
	a	b	a	b	a	b
Permagel	0.29	74.2	2.3	43.5	0.31	74.6
Attapulgite	0.38	66.3	0.25	40.1	0.29	71.4
Halloysite	0.27	18.7	0.36	22.2	0.39	14.9
Kaolinite	0.35	7.5	1.9	10.7	1.7	3.4
Kaolin N. F.	0.11	10.3	0.48	10.4	0.83	4.6

The hydrogen ion concentrations of the attapulgite-strychnine mixtures used in the determination of the adsorption isotherm were between 7.4 and 7.6. In order to study the effect of pH on the adsorption of strychnine by attapulgite, sulfuric acid was added to these mixtures to obtain various hydrogen ion concentrations. The use of buffers was not attempted, considering the possibility of interference with adsorption by the buffer salts. Other procedures for the determination of the isotherms were the same as described before. The isotherms obtained at three different pH ranges are shown in Fig. 5, and the corresponding values for the constants of the Langmuir equation are reported in Table IV.

TABLE IV.—CONSTANTS OF LANGMUIR'S EQUATION FOR THE ADSORPTION OF STRYCHNINE ON ATTAPULGITE AT DIFFERENT pH

pH	a	b
7.4–7.6	0.38	66.3
3.1–3.3	0.21	65.9
1.2–1.3	0.09	62.6

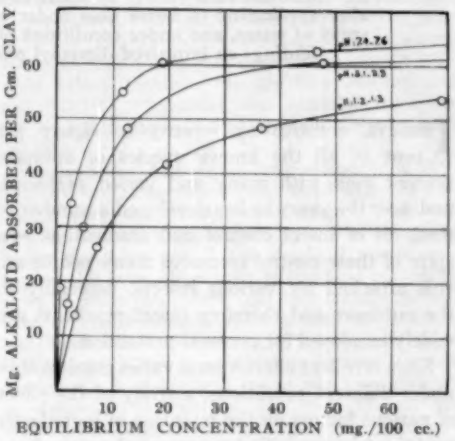

Fig. 5.—Adsorption isotherms at 24° C. for strychnine by attapulgite at different pH.

SUMMARY

1. A suitable method is described for the evaluation of the adsorptive power of adsorbents for alkaloids.

2. The adsorptive power of the different clay samples were in the same order for strychnine, atropine, and quinine.

3. Permagel, attapulgite, and halloysite were found to be better adsorbents for alkaloids than the sample of kaolin N. F. that was used.

4. An increase in hydrogen ion concentration was found to have a slight decreasing effect on the adsorptive ability of attapulgite for strychnine.

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The Retention of an Insect Repellent in Emulsified Bases at the Site of Application*

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Since repellent effectiveness varies considerably under different conditions, a study of the effect of various factors on the retention of carbon-14 labeled dimethyl phthalate contained in a variety of water-in-oil and oil-in-water emulsions was studied after application to horse skin under normal conditions, under exposure to a fine spray of water, and under conditions of sensible perspiration. On the basis of the findings an improved dimethyl phthalate cream formulation is presented.

INSECTS, constituting seventy to eighty per cent of all the known species of animals, present man with many and varied problems, and over the years he has developed a number of methods of insect control and eradication. In spite of these control measures many people are still attacked by various insects, especially in the outdoors and therefore insect repellents are widely employed for personal protection.

Since repellent effectiveness varies considerably under different conditions, a study of the effect of various factors on the retention of ingredients contained in emulsified creams at the site of application under several experimental conditions was made.

EXPERIMENTAL

In order to develop suitable techniques for determining the retention of materials applied on the skin and also to note the effects on retention of placing polar and nonpolar soluble substances in hydrophilic and lipophilic bases, experiments on the retention of sodium iodide and iodoform in o/w and w/o emulsified bases were conducted. In addition, experiments were conducted on the retention of dimethyl phthalate, one of the more common insect repellents. Isotope tracer techniques were employed to determine the quantity of material remaining on the skin at a given time. The isotopic materials employed were I^{131} labeled sodium iodide and iodoform and C^{14} labeled dimethyl phthalate.[1] The labeled iodoform was synthesized electrolytically from sodium iodide and acetone (1). The C^{14} labeled dimethyl phthalate was synthesized by Barker (3). The radiochemical purity of the iodoform and the dimethyl phthalate was established by ascending paper partition chromatography.

The species of animal used in this study was selected so that the characteristics and functions of the animal's skin would be as close as possible to that of man's. Upon surveying the field of experimental and domestic animals available, the horse was chosen as the one best suited for this type of work.

In preparing the skin for experimentation it was shaved, washed with soap, rinsed, dried, and marked off into 6.5-cm. squares. The samples of radioactive ointment (0.15 Gm. of NaI and iodoform and 0.1 Gm. of dimethyl phthalate) were weighed in paper weighing cups on a torsion balance and evenly applied to exposed circles of skin with a special spatula by placing a celluloid form, 6.5-cm. square, exactly within the marked boundaries on the skin. The form had a circular opening in the center of 2.9-cm. diameter. Five minutes after the ointments were applied, the initial activity of each circular skin area was determined by placing a lead shield 6.5-cm. square exactly within the marked boundaries on the skin and centering the end window of a Geiger tube attached to a counting rate meter over a shield. The counting shield of one-eighth inch lead has a circular center opening of 2.0-cm. diameter. Corrections for background, resolving time, and radioactive decay were made as required. Additional activity determinations were made at subsequent time intervals as specified in each experiment and the per cent retention calculated.

The three experimental conditions under which retention was studied were: normal (80° F. with no subsequent treatment), exposure to a fine spray of water, and sensible perspiration.

When determining the retention of materials on the skin exposed to a fine water spray, the initial activity was recorded and the areas sprayed with a fine mist of water for fifteen seconds. One minute after the spraying had ended, the activity of the skin areas was again measured. Five minutes after the end of the previous spraying the next fifteen-second water treatment was begun. Then the activity present was again noted, and the cycle was repeated for a total of four or five sprayings. The air temperature was kept constant at 80° F. and the water temperature was 70° F.

The procedure described by Collins (2) to induce and measure the quantity of sensible perspiration produced was used in determining the effect of perspiration on retention. The quantity of perspiration produced under the conditions of these experiments was determined eight times for each of the test sites to show the level of variation of perspiration flow and to insure reproducibility of results. In addition, the amount of sensible perspiration was determined in two adjacent test sites during each test as an added check. Reproducibility was quite satisfactory, the average quantity of perspiration collected from each of the eight test sights being 31.4, 29.4, 31.9, 33.0, 40.5, 37.1, 22.8, and 33.5 mg., respectively. In determining the effect of sensible

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[1] The isotopes used in this work were obtained on allocation by the United States Atomic Energy Commission.

perspiration, the test creams and two untreated control areas were rotated over the various skin areas during several different runs.

In order to compare the retention of sodium iodide and iodoform in an o/w and w/o emulsified ointment base, a series of bases was formulated and 5% sodium iodide and iodoform ointments were prepared. The formulas for the o/w and w/o ointments chosen for evaluation follow:

Oil-in-Water Emulsified Ointment

Stearic acid	22.00 Gm.
White petrolatum	3.00 Gm.
White wax	2.00 Gm.
Carbowax® 6000	7.00 Gm.
Distilled water	58.75 ml.
Glycerin	6.00 ml.
Triethanolamine	0.90 Gm.
Sodium benzoate	0.10 Gm.
Sodium iodide or iodoform	5.25 Gm.

Water-in-Oil Emulsified Ointment

White petrolatum	26.00 Gm.
White wax	9.40 Gm.
Mineral oil	21.25 ml.
Anhydrous lanolin	2.00 Gm.
Arlacel® 83	4.00 Gm.
Distilled water	37.00 ml.
Sodium benzoate	0.10 Gm.
Sodium iodide or iodoform	5.25 Gm.

The mean per cent retention of sodium iodide and iodoform in an o/w and a w/o emulsified ointment base under normal conditions at 80° F. was determined using five skin areas for each ointment. The results showed that the o/w base provided better retention than the w/o base and that sodium iodide was retained much better than the more volatile iodoform. Twenty hours after the application of the NaI in o/w and w/o emulsions, 81.5 and 53.4% respectively, were retained at the site of application. In the case of the iodoform applied in o/w and w/o emulsions, 38.2 and 13.1%, respectively, were retained.

The mean per cent retention of sodium iodide and iodoform in the emulsified ointments exposed to a fine spray of water was determined. For sodium iodide one ointment of each type (w/o and o/w) was tested at the same time on adjacent skin areas. Each ointment was applied alternately to one area and the other. The same procedure was followed with the iodoform ointments. From the data obtained it could be seen that the oily base provided better retention than the washable base and that the relative concentration of iodoform remained higher than that of sodium iodide. After five separate sprayings, 19.7 and 26.5% of NaI were retained in the o/w and w/o emulsions, respectively. In the case of the iodoform, 60.2 and 73.2%, respectively, were retained in the o/w and w/o bases.

The mean per cent retention of sodium iodide and iodoform in the emulsified bases when exposed to perspiration was also determined. Under conditions of perspiration for two hours, sodium iodide in the washable base was retained poorly (25.2%) while sodium iodide in the oily base was retained well (63.7%). Iodoform was retained better in the washable base (66.5%) than in the oily base (59.1%).

Several series of emulsified creams containing 40% dimethyl phthalate were formulated and the following were selected for evaluation: a standard cream, 1, to which the performance of others could be compared, an o/w, 2, and a w/o, 3, cream containing the same ingredients in identical quantities except for the emulsifiers, and four creams derived from a general formula where a fatty or lipophilic ingredient was varied at the 5% level. Of these four, creams 4 and 5 were identical except for one emulsifier. A stable w/o cream was very difficult to obtain. The formulas for these creams are presented in Table I.

A standard procedure was used in preparing all dimethyl phthalate creams. Ingredients A indicated above were melted over a water bath and warmed to 70° C. Ingredients B were heated slowly, added to A using a mechanical stirrer, and hand homogenized at 50–55° C. The C^{14} labeled dimethyl phthalate was incorporated into the creams by levigation.

The retention of dimethyl phthalate in cream 1 was determined at 40, 60, 70, and 80° F. In general retention is poorer at higher temperatures, but no specific pattern could be established over a range of 26–66% relative humidity.

The mean per cent retention of dimethyl phthalate in various creams (1 through 7) under normal conditions at 80° F. when exposed to a fine spray of water, and under perspiration conditions was de-

TABLE I.—COMPOSITION OF THE DIMETHYL PHTHALATE CREAMS

	Cream →	1	2	3	4	5	6	7	8
	Ingredients (Gm.)								
A	Stearic acid	14.0	13.0	13.0	13.0	13.0	13.0	13.0	5.0
	White wax	2.5	2.5	2.5	..	..	..	..	..
	White petrolatum	1.5	2.5	2.5	..	..	..	..	..
	Myvacet 5-00[a]	..	..	..	5.0	5.0	..	..	15.0
	Anhydrous lanolin	..	..	..	..	..	..	5.0	..
	Atlox® 1255[b]	2.0	2.0	..	2.0	2.0	2.0	2.0	2.0
	Arlacel® 83[b]	..	..	4.0	..	..	..	..	..
	DC 200 fluid, 1000 centistokes[c]	..	..	..	..	..	5.0	..	1.5
B	Distilled water	38.0	38.0	38.0	38.0	38.0	38.0	38.0	34.5
	Triethanolamine	0.5	0.5	..	0.5	0.5	0.5	0.5	0.5
	Myrj® 52[b]	..	1.5	..	1.5	..	1.5	1.5	1.5
	Tween® 60[b]	..	..	..	..	1.5	..	..	..
	Tween® 80[b]	1.5	..	..	..	..	..	..	..
C	Dimethyl phthalate (ml.)	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0

[a] Distillation Products Industries, Div. Eastman Kodak Co., Rochester, N. Y.
[b] Atlas Powder Co., Wilmington, Del.
[c] Dow-Corning Corp., Midland, Mich.

termined and the results are shown in Tables II, III and IV. The seven different creams were rotated over seven skin areas so that every cream was tested once on each area.

After the seven creams were compared under the three different conditions shown in Tables II, III, and IV, another cream (cream 8) was formulated (see Table I for ingredients) in an attempt to provide for improved retention. This cream was evaluated concurrently in separate experiments with cream 1, the standard cream, and cream 4, the best of the seven previously tested. The results obtained for cream 8 are included in Tables II, III and IV along with the additional results for creams 1 and 4.

After evaluating retention under the three experimental conditions and considering the properties of the various creams, creams 4, 5, and 8 which contained an acetostearin were found to be superior to the other preparations. Cream 8 had a firmer consistency before use but melted more readily upon application to the skin than did the other two creams. The major fatty ingredient in cream 8, Myvacet 5-00, had a melting point of 42–44° C. (7), compared to 55° C. for stearic acid, U. S. P. (8), the major fatty component of the other creams.

Considering the properties of the bases and the degree of retention of dimethyl phthalate they provided under the three experimental conditions, cream 8, containing 15% of an acetostearin, was superior to all others evaluated in this limited study. However, this base would have to be formulated containing other insect repellents, and field tests against mosquitoes would have to be conducted in order to evaluate it positively.

TABLE II.—MEAN PER CENT RETENTION OF DIMETHYL PHTHALATE IN VARIOUS CREAMS AT 80° F. UNDER NORMAL CONDITIONS[a]

Cream →	1	2	3	4	5	6	7	1	4	8
Time, Hr.	Concurrent Determinations									
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	72.0	72.8	74.4	73.0	71.6	66.7	72.1	72.4	73.4	76.1
2	55.2	56.1	47.4	56.1	55.6	48.8	52.2	56.9	57.5	61.4
4	33.0	35.8	39.3	41.2	40.6	31.3	34.7	37.7	42.4	47.2
6	20.6	23.3	27.3	31.3	30.6	21.7	23.4	21.1	34.9	38.5

[a] Each value is based on seven determinations.

From Table II it can be seen that cream 3, the w/o emulsion, and creams 4 and 5 which contained Myvacet 5-00, an acetostearin (4, 5, 6), were superior to the others after six hours had elapsed. Cream 8 provided the best retention.

TABLE III.—MEAN PER CENT RETENTION OF DIMETHYL PHTHALATE IN VARIOUS CREAMS AT 80° F. WHEN EXPOSED TO A FINE SPRAY OF WATER[a, b]

Cream →	1	2	3	4	5	6	7	1	4	8
No. Sprayings	Concurrent Determinations									
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	68.7	64.0	81.3	71.7	69.6	67.2	67.9	70.8	75.5	81.6
2	47.1	50.5	65.5	53.8	54.7	50.6	48.2	46.3	57.4	66.2
3	34.3	36.6	50.2	42.3	39.7	37.8	34.1	37.3	45.7	53.5
4	25.4	29.0	43.1	34.4	31.1	30.5	28.2	28.5	36.9	43.6

[a] Each value is based on five determinations.
[b] Varying the water temperature from 50 to 70° F. was previously shown to have no effect on retention in cream 1. Water at 70° F. and an air temperature of 80° F. were used.

From Table III it can be seen that the mean per cent retention of dimethyl phthalate in various creams when exposed to a fine spray of water remained in the same relative position as under normal conditions except that cream 3 (w/o) and cream 6 (silicone) improved their relative effectiveness. Thus, the w/o emulsion, cream 3 and cream 8, provided the best water resistance.

SUMMARY AND CONCLUSIONS

A procedure using radioactive tracer techniques has been developed for determining the retention of materials on the skin under three experimental conditions: normal, exposure to a fine spray of water, and conditions of sensible perspiration.

TABLE IV.—MEAN PER CENT RETENTION OF DIMETHYL PHTHALATE IN VARIOUS CREAMS UNDER PERSPIRATION CONDITIONS[a, b]

Cream →	1	2	3	4	5	6	7	1	4	8
Time, Hrs.	Concurrent Determinations									
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	60.1	59.9	65.5	64.3	65.1	62.1	61.7	46.4	51.1	53.3
2	33.9	36.9	46.0	44.5	44.7	40.1	40.3	28.9	36.6	38.4

[a] Each value is based on seven determinations.
[b] Room temperature ranged from 86 to 96° F.

Studies were made on the retention of sodium iodide, iodoform, and dimethyl phthalate in various emulsified ointment bases, including o/w and w/o types. The following conclusions were drawn.

1. An o/w base provided better retention of sodium iodide and iodoform than a w/o base under normal conditions.

2. The reverse of the above was true when the bases were subjected to fine water sprayings, the w/o base in this case showing the greatest retention.

3. Under conditions of perspiration, sodium iodide was retained poorly in the o/w base and quite well in the w/o base. The reverse was true for iodoform since the o/w provided greater retention than the w/o.

4. A w/o emulsion containing forty per cent dimethyl phthalate provides better retention at the site of application than an o/w cream containing the same ingredients in identical quantities except the emulsifiers.

On the basis of data obtained, an improved dimethyl phthalate cream containing fifteen per cent of the acetostearin was formulated, evaluated for its retention properties, and found to be superior to seven others which were studied. It is recognized that additional field tests against mosquitoes or other insects are necessary before a definite evaluation of this cream can be made.

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Countercurrent Analysis of Vitamin B_{12}*

By W. J. MADER and R. G. JOHL

Compounds similar to, but not identical to the cobalamins (cyanocobalamin or materials convertible to cyanocobalamin by cyanide treatment) occur in the commercial production of Vitamin B_{12}. Some of these noncobalamins show microbiological activity with the *L. leichmanii* organism and have similar ultraviolet absorption properties to cyanocobalamin. The application of the countercurrent separation followed by either spectrophotometric or microbiological assay is discussed together with the application to synthetic mixtures of cyanocobalamin and pseudocobalamin, noncobalamin materials from fermentation broths and commercial cobalamin concentrates. The results obtained are compared with the results of the more accurate radioactive tracer assay for Vitamin B_{12}. The advantages and disadvantages of the method are discussed.

NUMEROUS MICROORGANISMS (6, 7, 12, 14, 15) have been reported as elaborating vitamin B_{12} and/or vitamin B_{12} activity. *Clostridium tetanomorphum* (13) and various unidentified anaerobes have been shown to produce pseudovitamin B_{12}. There is no indication in the literature that the microbiologically active material in fermentation broths is totally cyanocobalamin or material convertible to cyanocobalamin. It is reasonable to assume that substances are present in commercial fermentation broths which are not cobalamins or materials convertible to cyanocobalamin. Unless care is taken in processing these broths it is reasonable to assume that noncobalamin microbiologically active material will show up in final products. The presence of microbiologically active noncobalamins in some commercial animal feed supplements has been reported by Chaiet (3). This indicates the necessity of using highly specific assays for the determination of the cobalamin and/or vitamin B_{12} content of commercial products.

Various microbiological procedures have been proposed such as the U. S. P. procedure using *L. leichmannii* (13), the *Lactobacillus lactis* Dorner (10), the *Escherichia coli* (10), and the *Ochromonas malhamensis* (5). The only one claimed to be specific is the *Ochromonas malhamensis*, which was reported in 1953, but to date has not been widely accepted.

* Received May 5, 1955 from Merck & Co., Inc., Chemical Division, Rahway, N. J.

Chemical assays of Boxer (2), Rudkin and Taylor (11), and Fisher (4) have been shown to be nonspecific (3); however, the radioactive tracer assay of Bacher (1) has been shown to be a highly specific and accurate assay for cyanocobalamin, or cobalamins easily convertible to cyanocobalamin. Of all the currently proposed assays, the radioactive tracer assay is the most accurate and specific.

The countercurrent distribution assay reported here is an excellent specific method to determine the purity or cyanocobalamin content of crystalline vitamin B_{12}, oral grade solids and simple solutions. One can determine the presence or absence of pseudocobalamin and other noncobalamin fractions. This method has been used for several years in the control of crystalline vitamin B_{12} purity.

PROCEDURE

I. **Conversion.**—Weigh accurately enough sample to give 50 μg. vitamin B_{12}, but not more than 0.500 Gm., into a 250-ml. Erlenmeyer flask. Add 50 ml. water, 5 ml. of 10% potassium cyanide and 5 ml. of 2% sodium nitrite. Adjust the *p*H to 4 using about 6 *N* acetic acid. Add a few glass beads and place the flask on a hot plate in the hood and boil for five minutes; a defoaming agent may be used. Remove the flask from the hot plate and allow to cool. Add 1 ml. of formaldehyde solution (37%) and mix thoroughly. Filter through a Buchner funnel and wash the residue with sufficient water to make up to 100 ml.

II. **Cresol-Butanol Extraction.**—To a 50 ml. aliquot of the clear filtrate add 5 ml. of 1:1 cresol-carbon tetrachloride and shake vigorously for one minute. Centrifuge and carefully draw off the lower cresol-carbon tetrachloride layer by means of a 10-ml. syringe. Repeat this extraction with another 5-ml. portion of cresol-carbon tetrachloride. To the combined cresol-carbon tetrachloride extracts add 4 ml. of 5 *N* sulfuric acid. Shake vigorously for about one minute and centrifuge. Carefully draw off the aqueous layer and discard. Repeat the washings at least two times or until the washings are colorless, or practically so. Now add 4 ml. of sodium bicarbonate (2%)-potassium cyanide (0.4%) solution, shake for one minute and centrifuge. Draw off the top aqueous layer by means of a 10-ml. syringe and discard. Repeat with another 4 ml. of the sodium bicarbonate-potassium cyanide solution. To the washed cresol-carbon tetrachloride solution add 20 ml. of chloroform and 5 ml. of butyl alcohol. Add 5 ml. of distilled water to the dilute mixture and shake vigorously for one minute. Centrifuge. Draw off the aqueous layer into a 25-ml. volumetric flask. Repeat the extraction with two additional 5-ml. portions of water. Make up to 25 ml. with water.

III. **Countercurrent Distribution.**—Shake reagent benzyl alcohol with an equal volume of distilled water until both phases are mutually saturated. Allow to settle and separate. Centrifuge before use to remove entrained liquid. The two liquids are referred to as the benzyl phase and the aqueous phase respectively.

Prepare eight 15-ml. centrifuge tubes, numbered 1 to 8. Transfer a 5-ml. aliquot of the 25-ml. solution prepared from the above extractions to tube 1. Pipet 5 ml. of aqueous phase into each of the other seven tubes. Add 5 ml. of benzyl phase to tube 1, stopper with a plastic closure, and shake gently for about one minute. Centrifuge, remove the lower (benzyl) phase with a hypodermic syringe and transfer it to tube 2. Add 5 ml. of fresh benzyl phase to tube 1. Stopper and shake both tubes as before and centrifuge. Transfer the benzyl phase from tube 2 to tube 3, the benzyl phase from tube 1 to tube 2, and add 5 ml. of fresh benzyl phase to tube 1. Stopper and shake the three tubes and centrifuge. Continue the process until all eight tubes contain two phases in equilibrium. If difficulty in separating the phases is encountered, centrifuge for about five minutes at high speed.

Dilute each aqueous phase from the eight tubes to 10 ml. To each benzyl phase from the eight tubes add 5 ml. distilled water and 5 ml. of carbon tetrachloride. Shake, centrifuge, and remove the upper aqueous layer. Dilute each of these aqueous solutions to 10 ml.

Analysis of Phases.—The vitamin B_{12} content of the phases can be determined either microbiologically, spectrophotometrically or colorimetrically.

Calculation of Results.—The distribution coefficient, K, for cyanocobalamin is

$$K = \frac{C\ (\text{upper})}{C\ (\text{lower})} = 1.2$$

for the system water saturated with benzyl alcohol and benzyl alcohol saturated with water. The distribution of cyanocobalamin in each tube following an eight tube distribution is listed in Table I.

$$\%\ \text{Vitamin } B_{12} = \frac{\text{Concentration } B_{12} \text{ in Tube 4/Wt. of Sample}}{0.291} \times 100$$

since 29.1% of the cyanocobalamin will concentrate in tube 4.

Having calculated the vitamin B_{12} content assuming only B_{12} was determined on analysis of tube 4, one can calculate the amount of impurity in the other seven tubes. The observed fraction minus the product of the theoretical vitamin B_{12} fraction (Table I) for the tube in question by the vitamin B_{12} content of the sample represents impurity. The mathematical calculations involved are adequately described in Organic Analysis (8).

APPLICATIONS

Red Pigments.—Several red pigments have been isolated from crude fermentation broths. These pigments were not purified and their structure is unknown (9). In Table II, the spectrophotometric data are tabulated and compared with an authentic sample of cyanocobalamin. The wavelengths λ_1, λ_2, and λ_3 represent the major peaks for cyanocobalamin and λ_4 a minimum. These pigments could easily be mistaken for cyanocobalamin if assayed spectrophotometrically. Figure 1 shows the countercurrent distribution of these pigments. The dis-

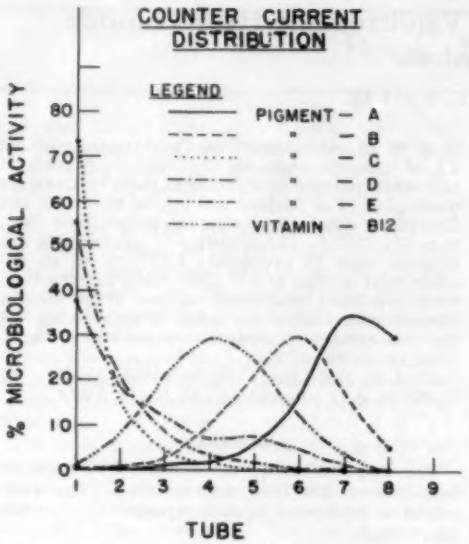

Figure 1.

TABLE I.—THEORETICAL DISTRIBUTION OF CYANOCOBALAMIN

SYSTEM: WATER AND BENZYL ALCOHOL. MUTUALLY SATURATED

Tube No.	Fraction in Aqueous Phase	Fraction in Both Phases
1	0.008	0.0144
2	0.045	0.0838
3	0.114	0.210
4	0.159	0.291
5	0.132	0.242
6	0.066	0.121
7	0.018	0.0337
8	0.002	0.0040

TABLE II.—SPECTROPHOTOMETRIC DATA

Sample	λ_1	λ_2	λ_3	λ_4
Cyanocobalamin	361	550	278	430
Pigment A	358	540–550	282	430
B	361	550–552	284	430
C	357	550	280	430
D	356–361	540–542	280	430
E	...	538	280	430

TABLE III.—SYNTHETIC MIXTURES OF CYANOCOBALAMIN AND PSEUDOCOBALAMIN

Theory		Found
% Cyano	% Pseudo	% Cyanocobalamin
100	..	101
90	10	88
70	30	67

tribution on pigment E indicates the presence of about 20 to 30% cyanocobalamin. These distributions are based on microbiological activity.

Pseudocobalamin.—Crystalline pseudocobalamin with a distribution constant of 8, isolated by Chaiet (3) from a commercial animal feed supplement was used to prepare synthetic mixtures containing 10% and 30% pseudocobalamin with the remainder cyanocobalamin. After countercurrent distribution the phases were assayed microbiologically by the U. S. P. procedure and the results tabulated in Table III.

TABLE IV.

Sample	Countercurrent Distribution Microbiological, μg. Cobalamin/Gm.	Tracer, μg. Cobalamin/Gm.	Difference, %
A	940	960	−2
B	900	950	−5
C	930	975	−5
D	1030	1040	−1
E	1060	1040	−2
F	1020	1020	0
G	990	1025	−3

TABLE V.—VITAMIN B_{12} INJECTION

Sample	Labeled Content	U. S. P. Spectrophotometric Assay	CCD Assay	% of U. S. P.
A	15	15.5	15.4	99.4
B	15	16.0	16.0	100.0
C	1000	1178	995	84.5
D	1000	903	879	97.3
E	1000	925	650	70.0

Crude Cobalamin Concentrates.—Seven commercial cobalamin concentrates and oral grade solids were analyzed by the countercurrent distribution microbiological method and the results compared to the analysis by the radioactive tracer method. The agreement is excellent. The results are tabulated in Table IV.

Vitamin B_{12} Injection.—Five commercial samples of Vitamin B_{12} injections were analyzed by both the U. S. P. XIV spectrophotometric method and by the countercurrent distribution procedure (Table V).

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A Note on the Preparation of Vanillamidine Hydrochloride Hemihydrate*

By DONALD F. WALKER

THE PUBLICATION of clinical experience with ethyl vanillate in cases of disseminated histoplasmosis (1) attracted attention to the desirability of preparing the amidine analog of vanillin.

A procedure has been described by Pearl (2) for preparing ethyl vanillate from vanillin which includes in the synthetic route vanillonitrile and the iminoester derived from it. It appeared likely that this iminoester would be readily converted to the amidine by reaction with ammonia (3).

The compound was ineffective when tested against *Histoplasma capsulatum* and other representative pathogenic fungi.[1]

EXPERIMENTAL

Vanillamidine Hydrochloride Hemihydrate.—Two and one tenth grams of ethyl 3-methoxy-4-hydroxybenzimidate hydrochloride were suspended in 25 ml. of anhydrous ethanol and treated with 5.3 ml. of ethanolic ammonia (0.17 Gm.). A granular tan solid separated on standing at room temperature overnight. The mixture was heated to boiling and filtered to obtain an insoluble residue, 0.58 Gm., m.p. 254–255°.[2] The filtrate was diluted with anhydrous ether to precipitate 1.15 Gm. of an off-white solid melting at 268–270°. The two fractions were combined and recrystallized from ethanol diluted with anhydrous ether after filtering. A light tan crystalline product was obtained in 64.5% yield, m. p. 268–270°.

Anal.—Calcd. for $C_8H_{10}N_2O_2 \cdot HCl \cdot {}^1/_2H_2O$; C, 45.39; H, 5.71. Found: C, 45.24; H, 5.92.[3]

SUMMARY

The preparation of vanillamidine hydrochloride hemihydrate has been accomplished. The compound is ineffective against representative pathogenic fungi.

* Received June 20, 1955, from the Research Laboratories, Parke, Davis & Company, Detroit, Mich.

[1] Antifungal screening was performed in this laboratory by Dr. A. B. Hillegas and associates.

[2] All melting points are uncorrected.

[3] Microanalysis was performed in this laboratory by C. E. Childs and associates.

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A Note on the Optical Rotation of Narcotine*

By FREDERICK W. BOLLINGER

RECENT INTEREST (1–3) in narcotine as an antitussive agent prompts us to report a correction of the value for the optical rotation of narcotine in benzene. The oft-quoted value of Dott (4), $[\alpha]_D = -229°$ (c, 1.59 in benzene) could not be repeated. Instead we obtained $[\alpha]_D^{25} = -147°$ (c, 1.59 in benzene) and $[\alpha]_D^{25} = -147°$ (c, 1 in benzene). For the above determinations narcotine Merck was crystallized twice from aqueous acetone, m. p. 176°, lit. (5) m. p. 176°. The purity of this sample of narcotine estimated by phase solubility was 99.9% and its optical rotation reproduced literature values as follows: $[\alpha]_D^{25} = -200°$ (c, 1 chloroform), lit. (6) $[\alpha]_D = -199.85°$ (c, 2–3.3 chloroform); $[\alpha]_D^{25} = +57.5°$ (c, 2 0.1*N* H_2SO_4), lit. (7) $[\alpha]_D^{25} = +57.25°$ (c, 2 1% H_2SO_4) and $[\alpha]_D^{25} = -146°$ (c, 2 toluene), lit. (7) $[\alpha]_D^{25} = -148.75°$ (c, 2 toluene). The close agreement of the values for the optical rotation of narcotine in benzene and in toluene was to be expected.

* Received June 10, 1955, from the Research Laboratories, Chemical Division, Merck & Co., Inc., Rahway, N. J.

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Book Notices

Rutin and Related Flavonoids. JOHN Q. GRIFFITH, JR., CHARLES F. KREWSON, and JOSEPH NAGHSKI. Mack Publishing Company, Easton, Pa., 1955. 13 + 275 pp. 16 x 23.5 cm. Price $7.50.

The greater part of this book is devoted to a review of the literature on the effect of rutin upon the hemorrhagic complications of hypertension. In one chapter, however, the chemistry of rutin, sources, methods of preparation from various plants, and criteria of quality are competently summarized by Doctors Krewson and Naghski of the Eastern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture of Philadelphia.

The main purpose of the book is to present an evaluation of the clinical research of Dr. J. Q. Griffith, Jr., of Philadelphia, and to review the pharmacological and clinical work of others on rutin, quercetin, and other flavonoids. The use of rutin in the treatment of capillary fragility is especially emphasized. The drug has also been investigated clinically and pharmacologically for possible application in the treatment of retinal hemorrhage, purpura, hereditary telangiectasia, radiation sickness, glaucoma, hemophilia, and drug idiosyncrasy. The results of these studies are adequately summarized. In general, the evidence both pro and con of the therapeutic value of rutin has been stated objectively. It should be of especial interest to practicing physicians, pharmacologists, and medical and pharmaceutical scientists employed in the pharmaceutical industry.

Rutin and Related Flavonoids is documented with more than eight hundred references to the original literature. In addition it presents for the first time the results of clinical studies on rutin by Dr. Griffith, extending over a nine-year period, during which an imposing array of evidence was accumulated indicating that rutin has been effective in correcting capillary fragility. A more recent three-year study is reported which indicates that quercetin, the aglycone of rutin, is even more effective than the parent compound in correcting conditions for which the latter has also been found to be useful.

The Roger Adams Symposium. Papers presented at a symposium in honor of Roger Adams at the University of Illinois, September 3 and 4, 1954. John Wiley & Sons, Inc., New York, 1955. ix + 140 pp. 15.5 x 23.5 cm. Price $3.75.

One of the best known names in the field of organic chemistry is that of Roger Adams, who has contributed such a large share to the growth of this subject through his own research and by training many students for successful careers in research fields. It was therefore quite fitting that special tribute should be paid to him upon his retirement as head of the Department of Chemistry at the University of Illinois in September, 1954, after nearly forty years of service to that institution. This tribute, planned by his present and former students took the form of a symposium covering a two-day period during which papers were presented by six well-known chemists who received their indoctrination in organic chemical research under Dr. Adams' direction. The symposium started by a brief review of the career of Dr. Adams, which was presented by Dr. Ernest H. Volwiler, President of the Abbott Laboratories. This was followed by papers entitled: "Steric Effects in Dyes," by Wallace R. Brode, Associate Director, National Bureau of Standards; "The Structure of Gliotoxin, a Sulfur-Containing Antibiotic Substance," by John R. Johnson, Professor of Chemistry, Cornell University; "The Structure of Nepetalic Acid," by Samuel M. McElvain, Professor of Chemistry, University of Wisconsin; "Chemistry of Flavylium Salts, Reactions with Amines," by Ralph L. Shriner, Professor of Organic Chemistry and Head of the Department of Chemistry, State University of Iowa; and "Some Chemical Studies on Viruses," by Wendell M. Stanley, Professor of Biochemistry and Director of the Virus Laboratory, University of California. In this book the papers presented to the Symposium are reproduced and quite properly the book itself is dedicated to the man for whom the symposium was designed to honor.

The Nucleic Acids. Chemistry and Biology, Vol. 2. ERWIN CHARGAFF and J. N. DAVIDSON, Editor. Academic Press Inc., New York, 1955. xi + 576 pp. 16.5 x 23.5 cm. Price $14.50.

Volume I of The Nucleic Acids was described previously [THIS JOURNAL, **44**, 453,(1955)]. Volume II follows the design of Volume I and includes thirteen chapters. The scope of Volume II can best be visualized from a list of the titles of the several chapters. These are as follows: The Nucleic Acid Content of Tissues and Cells; Cytochemical Techniques for Nucleic Acids; The Isolation and Composition of Cell Nuclei and Nucleoli; The Deoxyribonucleic Acid Content of the Nucleus; Nucleic Acids in Chromosomes and Mitotic Division; The Cytoplasm; Biosynthesis of Pentoses; Biosynthesis of Purines and Pyrimidines; Biosynthesis of Nucleosides and Nucleotides; Biosynthesis of Nucleic Acids; The Metabolism of the Nucleic Acids; The Biological Role of the Deoxypentose Nucleic Acids; and The Biological Role of the Pentose Nucleic Acids. Each chapter has been contributed by a specialist in the field covered. With the publication of Volume II there is now available a comprehensive presentation of all developments in the field of nucleic acids, which will serve as an adequate reference and source of information on the subject up to the beginning of 1955. The editors have done an excellent job of editing the contributed chapters and are to be congratulated upon their success. Volume II of The Nucleic Acids, like Volume I, is thoroughly documented and provided with adequate author and subject indexes. The printing and binding are of excellent quality and the book can be recommended to anyone interested in the chemistry and physiology of the nucleic acids.

The Biologic Effects of Tobacco. ERNEST L. WYNDER, Editor. Little, Brown and Company, Boston, 1955. xiii + 215 pp. 14.5 x 21 cm. Price $4.50.

The effect of tobacco on human health has been a controversial issue for many years. During the past several years contradictory reports have appeared in the literature concerning the relationship of the use of tobacco to certain diseases, with particular emphasis upon the incidence of lung cancer in cigarette smokers, as compared with nonsmokers. *The Biologic Effects of Tobacco* is designed to present the facts about the possible relationship of tobacco and tobacco smoke to human health. It summarizes more or less objectively the evidence and documents it by references to the original literature on the subjects covered. Tobacco consumption has been most frequently linked to diseases of the cardiovascular system, the gastrointestinal tract, allergies, and certain types of cancers. The data assembled are presented in seven well-written chapters. In the first chapter the chemistry of tobacco is reviewed, and of special interest here is a list of compounds which have been reported as present in tobacco smoke. In the second chapter on pharmacology, compounds of pharmacologic interest in tobacco smoke, with special emphasis upon the properties of nicotine, are discussed. Other chapters which follow cover the physiologic effects, pharmacologic considerations, and clinical data on the effects of tobacco on the cardiovascular system, the gastrointestinal tract, and allergies. In one chapter entitled "Neoplastic Diseases," Dr. Ernest L. Wynder, Head, Section of Epidemiology, and Associate, Sloan-Kettering Institute for Cancer Research, summarizes previously published clinical and statistical reports dealing with the role of cigarette smoking in the causation of lung cancer and cancer of the larynx, tongue, buccal mucosa, floor of the mouth, gums, and esophagus. From a study of these data, he concludes that cigarette smoking is a causative factor in lung cancer, and increases significantly the risk of cancer development in the buccal cavity. The seventh and final chapter entitled "Cause and Effect," by E. Cuyler Hammond, Director of Statistical Research of the American Cancer Society, summarizes the evidence, based upon epidemiologic research, which strongly indicates a definite relationship between cigarette smoking and the incidence of certain neoplastic diseases. The book is provided with an adequate subject index and with an author index which adds greatly to its value as a reference to the several ramifications of the subjects discussed.

Electrochemistry in Biology and Medicine. THEODORE SHEDLOVSKY, Editor. John Wiley & Sons, Inc., New York; Chapman & Hall, Ltd., London, 1955. xii + 369 pp. 16.5 x 23.7 cm. Price $10.50.

This book is based upon the papers presented at a symposium on electrochemistry in biology and medicine sponsored by the Electrochemical Society in April, 1953. The subjects covered include membranes, nerve and plant cells, biologically important ions, and applications of polarography, electrocardiography, and electroencephalography in medicine. Experimental data, tabulations, diagrams, and pictures increase the value of the text; and the references and general index make it an excellent addition to the medical and pharmacological research libraries.

The Hypophyseal Growth Hormone, Nature and Actions. RICHMOND W. SMITH, OLIVER H. GAEBLER, and C. N. H. LONG, Editors. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1955. xv + 576 pp. 16.5 x 23.5 cm. Price $12.

This is a record of the complete proceedings of an international symposium held in October, 1954. The book was prepared for publication with the view that the recorded data, methods, and speculations pertaining to the research development on the hypophyseal growth hormone could be made available quickly in this accelerating field of study. Growth hormone has as yet shown little clinical promise but much is being learned of its functions in the basic biological and medical sciences. The printing, format, and illustrations in the book are excellent; but the absence of a general index is a regrettable omission in a compilation that is a splendid review and reference volume in its field.

Microbiology. 2nd ed. By FLORENE C. KELLY and K. EILENE HITE. Appleton-Century-Crofts, Inc., New York, 1955. xi + 615 pp. 17 x 23.5 cm. Price $7.50.

The first edition of this book was published in 1949 and was reviewed in THIS JOURNAL, **39**, 59 (1950). The advances in microbiology since 1949 include increased knowledge of bacterial genetics, cellular structure, biochemistry, metabolism, immunology, bacterial viruses, as well as new disease agents and antibiotics. The authors have incorporated much of this information in the second edition without going into great detail. An additional section on staining methods and formulas for stains, indicators, culture media, and tests for biochemical activities is appended. A chronological list of discoverers and their contributions starting with Roger Bacon (1267) and ending with Albert Calmette (1924) is given. The book is a suitable text on introductory microbiology for pharmacy students and general science courses.

Biochemistry. An Introductory Textbook. By FELIX HAUROWITZ. John Wiley & Sons, Inc., New York, 1955. 16 + 485 pp. 15.5 x 23.5 cm. Price $6.75.

Dr. Haurowitz's book can be recommended as an excellent and original introduction into the field of general biochemistry. Emphasis is placed on intermediary metabolism and on the mechanism of metabolic reactions, and recognition is given to the fact that the truly significant problems of biochemistry are those of living cells and tissues. The voluminous material which resulted from research in the field of proteins, particularly during the last twenty years, has been carefully selected and treated in such a way that its study is considerably facilitated. It deserves mention that the author stresses the act that there is no need for the student to memorize

the very complicated chemical formulas contained in the book. While the book limits itself strictly to its avowed purpose of serving as a text, and does not aspire to be a reference book at the same time, the list of review articles appended to each chapter will be helpful to the scholar working in a related field as well as to the reference librarian who has to supply such material or peruse it himself to get an over-all view of certain aspects of the chemistry and biology of proteins.

General State Food and Drug Laws. Food Law Institute Series. Annotated. By DAVID H. VERNON and FRANKLIN M. DEPEW. Commerce Clearing House, Inc., Chicago, 1955. xii + 804 pp. 17.5 x 25 cm. Price $17.50.

This is the seventh in the Food Law Institute Series of books dealing with laws relating to foods and drugs. The previous books in the series have been reviewed from time to time in this Journal. In this seventh book the general state food and drug laws are compiled and annotated by reporting cases based upon them. In this book will be found the full texts of general state laws relating to the manufacture, packaging, labeling, distribution, advertising, and sale of foods, drugs, cosmetics, and therapeutic devices. Many leading court decisions interpreting and applying these laws are summarized. These statutes and cases relate not only to adulteration, misbranding and false advertising, but also to standards, the introduction of new drugs, seizure powers, injunctions, and similar points of general state law. The relevant law and case material are conveniently grouped in alphabetical order by jurisdiction for the forty-eight states, the District of Columbia, Hawaii, Alaska, and Puerto Rico. A comprehensive table of contents and a detailed index, pin pointing the data by subject and state, contribute greatly to the usefulness of this excellent book. The printing and binding are of the same design and good quality of its predecessors and its publication will be welcomed both by lawyers and laymen who are connected with either the food or the drug industries.

Special Federal Food and Drug Laws. Food Law Institute Series. Annotated. By THOMAS W. CHRISTOPHER and CHARLES WESLEY DUNN. Commerce Clearing House, Inc., Chicago, 1955. xiii + 1,334 pp. 17.5 x 27.5 cm. Price $26.50.

The full texts of the Federal Food, Drug, and Cosmetic Act and the Federal Trade Commission Act are usually kept readily accessible by everyone concerned with the federal food and drug laws. Frequently, however, the full texts of numerous special federal laws dealing with foods and drugs, with information on their legislative history and related regulations may not be so readily available.

In this sixth book in the Food Law Institute Series, all special laws have been compiled for the first time within the covers of a conveniently bound book. Here will be found complete information on some twenty special federal food and drug laws such as the Public Health Act, as it relates to biological products; the Virus-Serum-Toxic Act of 1913; the Federal Caustic Poison Act; The Federal Insecticide, Fungicide, and Rodenticide Act; and the Narcotic Drugs and Marihuana Act; to mention a few of particular pharmaceutical interest. In the food field will be found the Meat Inspection Act; the Packers and Stockyards Act of 1921; and numerous others of special interest in this highly important field. The value of the book is enhanced by annotations to cases which illustrate the scope and purpose of the law in question.

The Law of Drugs and Druggists. 4th ed. By WILLIAM R. ARTHUR. West Publishing Co., St. Paul, 1955. xxxi + 399 pp. 19.5 x 26 cm. Price $6.

In his review of the third edition of this work (1947), Steven Wilson stated that " 'Law of Drugs and Druggists' is perhaps the only book which attempts to deal with the entire subject of pharmaceutical law, and it approaches the task in a very effective manner." This statement is just as true now as it was then. The uniqueness of the work makes it indispensable for anyone concerned with the legal problems of pharmacy. The uniqueness of the book also may serve as an excuse for its real weakness, which lies in the fact that it is meant to be a book of general information and reference, and a textbook for pharmacy students at the same time. The result is a certain lack of continuity of the "text," which largely consists of digests or excerpts from cases and statutes that might well have been worked into a continuous *text* for the benefit of the student. On the other hand, the interspersed review questions certainly do not contribute to the reference value of the book. The new edition follows the pattern set by the preceding ones; that is, Part I discusses state and local laws while Part II is given over to federal statutes and regulations. Within the two parts of the book, numerous chapters have been shifted around for reasons unexplained and not immediately evident to this reviewer.

All the material has been brought up to date. Important new decisions have been included. Of particular timeliness are two decisions holding that fluoridation of city drinking water is a valid exercise of police power. In the field of vitamins, a syllabus prepared by the Minnesota Supreme Court (1953) may be mentioned. It construes vitamins to be drugs within the statute restricting the sale of drugs. There is much that is new in such varied fields as state boards, hormones, liability, negligence, etc.

The format of the book has been improved considerably. The size of the pages has been enlarged so that each page takes two columns, thus facilitating the use and reading of the work. As we said at the outset of our discussion, this work outlining the legal pitfalls and angles to the retail pharmacist makes it a valuble reference for the practitioner.

Physiopathologie du Potassium. Colloques Internationaux de Centre National de la Récherche Scientifique, Paris, June 14–18, 1954. Service des Publications du Centre National de la Recherche Scientifique, Ministre de l'Éducation Nationale, Paris, 1954. 170 pp. 21 x 27 cm. Price 2,000 fr.

This French book is a compilation of a series of papers by competent authors who present well-authenticated discussions on related phases of the role of potassium in physiopathology. The papers

are titled: Introduction to the Biochemistry of Potassium; Utilization of Radioactive Potassium in Biology; Potassium of the Extracellular Fluid; Potassium and Sympathetic Ganglions; Potassium and Parasympathetic; Action of Potassium on the Central Nervous System; Pharmacodynamic Modification of Muscular Action by Potassium; Role of Potassium in Neuromuscular Transmission; Role of Potassium in the Excitability of Nerve and Muscle Fiber; Effects of Modification of Serum and Tissue Potassium on the Electrocardiogram; Role of Potassium in the Pathogenesis of Metabolic Alkalosis; Potassium in Digestive Disturbances of Infants and Children; The Proportion of Plasma Potassium During Anuric Nephritis; Conditions of Appearance of Hyper- and Hypopotassemia in the Course of Renal Insufficiency; Potassemia in Chronic Nephritis; Repercussion of Surgery on Potassium Equilibrium; Role of Potassium in Periodic Familial Paralysis; Potassium and Cortico-suprarenols; Disturbance of Potassium Metabolism in Diabetic Coma; and Treatment of Hyper- and Hypopotassemia.

BOOKS RECEIVED

The Pharmacopeia of the United States of America. 15th rev. By authority of the United States Pharmacopoeial Convention, Inc. meeting at Washington, D. C., May 9 and 10, 1950. Prepared by the Committee of Revision and published by the Board of Trustees. Mack Publishing Company, Easton, Pa., 1955. lii + 1178 pp. 15.5 x 23.5 cm. Price $10. domestic and $10.50, foreign.

Annotated Bibliography of Vitamin E. Vol. III. Compiled by PHILIP L. HARRIS and WILMA KUJAWSKI. Published by Eastman Kodak Company, Rochester, New York, 1955. 182 pp. 21 x 27.5 cm. Price $3.

Ageing—General Aspects, Vol. 1. Ciba Foundation Colloquia on Ageing. G. E. W. WOLSTENHOLME, MARGARET P. CAMERON, and JOAN ETHERINGTON, Editors. Little, Brown and Company, Boston, 1955. xi + 255 pp. 14.5 x 21 cm. Price $6.75.

Citizens Advisory Committee on the Food and Drug Administration. Report to the Secretary of Health, Education, and Welfare. June, 1955.

The Human Adrenal Cortex, Vol. 8. Ciba Foundation on Endocrinology. G. E. W. WOLSTENHOLME, MARGARET P. CAMERON, and JOAN ETHERINGTON, Editors. Little, Brown and Company, Boston, 1955. xv + 665 pp. 14.5 x 21 cm. Price $10.

New and Nonofficial Remedies, 1955. The Council on Pharmacy and Chemistry, American Medical Association. J. B. Lippincott Company, Philadelphia, 1955. xlviii + 653 pp. 13.5 x 18.3 cm. Price $3.35.

The Genus Nicotiana. Vol. 16. By THOMAS HARPER GOODSPEED. Chronica Botanica Company, Waltham, Mass., 1954. Illustrated. xxii + 536 pp. 16.5 x 25 cm. Price $12.50.

Prüfung und Verarbeitung von Arzneidrogen. Vol. 2. Verarbeitung Allgemeine und spezielle Methoden. By Dr. FRITZ GSTIRNER. Springer-Verlag, Berlin/Göttingen/Heidelberg, 1955. v + 249 pp. 16 x 24.5 cm. Price DM 24.

Methods for Evaluation of Nutritional Adequacy and Status. A Symposium sponsored by the Quartermaster Food and Container Institute. Washington, 1954.

Clinical Toxicology. By CLINTON J. THIENES and THOMAS J. HALEY. Lea & Febiger, Philadelphia, 1955. 457 pp. 13.5 x 20 cm. Price $6.50.

A Dictionary of Terms in Pharmacognosy and Other Divisions of Economic Botany. By GEORGE M. HOCKING. Charles C Thomas, Springfield, Ill., 1955. xxv + 284. 16.5 x 24.5 cm.

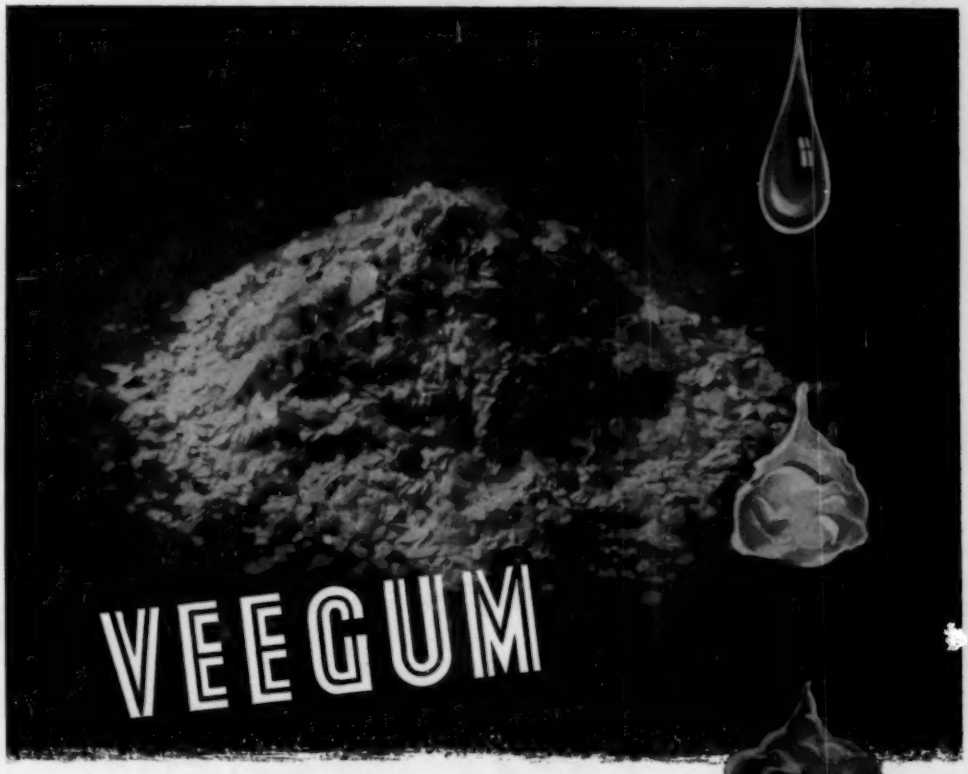
Methoden der organischen Chemie. Vol. 3, Part 2, Physikalische methoden. (HOUBEN-WEYL). Georg Thieme Verlag, Stuttgart, 1955. XXVIII + 1078 pp. 18.5 x 26 cm. Price $44.30.

Introduction to Chemical Pharmacology. By R. B. BARLOW. John Wiley & Sons, Inc., New York, 1955. xiv + 343 pp. 15 x 22.3 cm. Price $6.25.

Apotekens Register över Standardförpackade Läkemedel 1955. Compiled by Apotekarsocietetens Laboratorieavdelning Apotekens Kontrollaboratorium. Victor Pettersons Bokindustri Aktiebolag, Stockholm, 1955. 502 pp. 18.5 x 23.5 cm.

Catalysis. Vol 3. Hydrogenation and Dehydrogenation. Edited by PAUL H. EMMETT. Reinhold Publishing Corporation, New York, 1955. vii + 504 pp. 16 x 23.5 cm. Price $12.

An Arco Career Book. Pharmacist License Test. By MOSES A. LEVINE. Arco Publishing Company, New York, 1955. 20 x 26 cm. Price $3.50.



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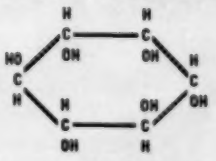

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